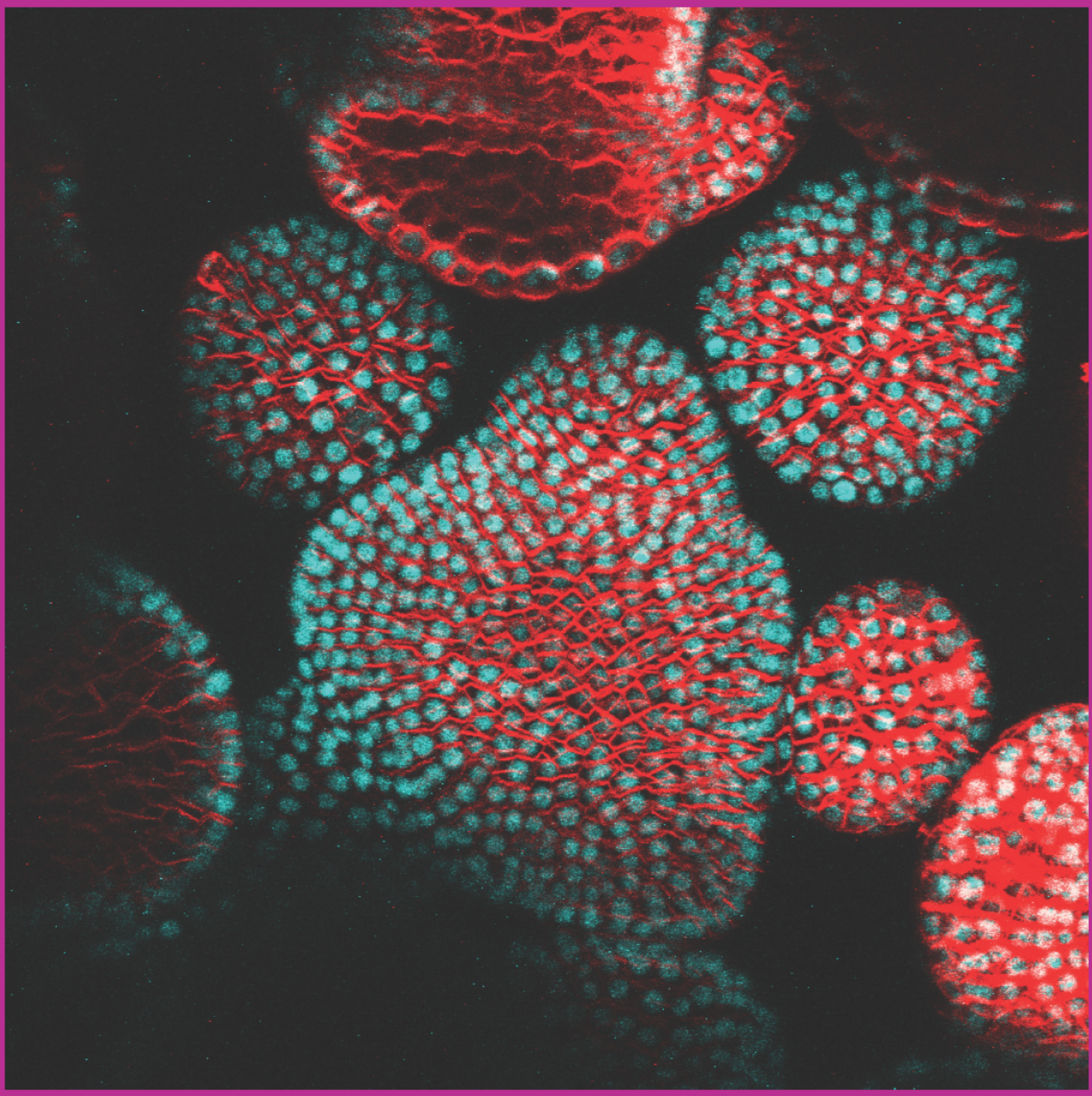

CALTECH Biology Annual Report 2006

Legend for the Front Cover Illustration

A confocal projection of an *Arabidopsis thaliana* meristem (growing tip) and initiating flower buds. The plasma membranes outlining the cells have been stained with FM4-64 (red) and the nuclei in the outer layer have been marked with a nuclear localized CFP (cyan). (Adrienne Roeder, Ph.D. – Elliot Meyerowitz's lab)

Legend for the Back Cover Illustration

Ellipsoid rendering of the diffusion tensor for a mid-coronal slice, showing the corticospinal tract and corpus callosum. Colorscale indicates direction of principle eigenvector of the tensor (red = LR, green = AP, blue = SI). Data is acquired from a normal adult human subject using the CBIC 3T scanner. (J. Michael Tyszka, Ph.D. – Member of the Professional Staff, Scott E. Fraser's Lab)

Division of Biology

California Institute of Technology

Pasadena, California

Annual Report
2005 - 2006

BIOLOGY - 2006

Yolanda Duron, Annual Report Coordinator

Research Reports

Biological research summarized in this report covers the time period from June, 2005 through July, 2006. The annual report is not intended to serve as an official forum, since some portions of the research listed in this report have not yet been published. When referring to an individual abstract(s), special permission must be obtained from the investigator.

References to published papers cited throughout the report are listed at the end of each individual research report.

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FACILITIES

INTRODUCTION

100 Years Ago: 1906

The 1906 Throop Institute Bulletin notes that the Shop and Laboratory Fee for Biology, payable at the beginning of each term, is $1.00. This was in addition to annual tuition fee of $85. The Biology faculty in 1906 was Professor of Biology Joseph Grinnell, and Lecturer Ernest Bryant Hoag. Grinnell was a noted mammalogist and ornithologist who later made important contributions to ecology and field biology; Grinnell Mountain in the San Bernardino Range is named after him.

75 Years Ago: 1931

In May, 1931 the then three-year old Division of Biology lost one of its early members in a tragic accident. Professor Karl Belar, who joined the Division in 1929 from the Kaiser Wilhelm Institute for Biology in Berlin, died in an automobile accident near Victorville, on a trip to the California desert that he had come to love. He was a world-renowned cytologist of the protozoans, studying what we would call now chromatin dynamics. At the time of his death he was 35 years old.

The biochemist Hugh Huffman was hired as an assistant professor in 1931, and remained on the faculty as an assistant professor until 1943. His specialty was calorimetric measurements of biologically important molecules. He left the Institute in 1943 to establish a laboratory of thermodynamics at the U.S. Bureau of Mines, in Bartlesville, Oklahoma.

50 Years Ago: 1956

From Biology 1956, the 1956 Annual Report:

"In addition to 20 regular members of the faculty [there were] eight Research Associates and 85 Postdoctoral Research Fellows. There were 16 undergraduate students in the biology option and 25 graduate students working toward the Ph.D. degree in the Division."

"The virologists and biophysicists of the Division are now moved into the newly completed Church Laboratory and for the first time have adequate facilities for their research."

"Investigations of gene nature and gene function continue to be carried out on a variety of organisms: viruses, bread mold, corn, fruit fly and several other plants and animals… A problem that is currently receiving much attention by geneticists in several laboratories is the so-called fine structure of the gene. It seems probable that in all organisms primary information is carried in nucleic acid molecules in the form of sequences of four subunits called nucleotides…"

25 Years Ago: 1981

From the CALTECH Biology Annual Report 1981:

"1980-81 was year of transition for the Division of Biology. Professor Leroy Hood took over the Division Chairman's responsibilities in, September, 1980… Lee faces the challenges of finding funding for new programs in an era of budget-cutting…"

"In January, 1981, we welcomed Mary Kennedy, who became Assistant Professor of Biology… In the fall of 1981, Barbara Wold, who completed her Ph.D. here in 1978, will return as Assistant Professor of Biology."

"In 1981 we lost two senior members of our faculty. Professor James Bonner retired at the end of the academic year, after more than 50 years of association with the Division of Biology as student and faculty member… On March 10, 1981, Professor Max Delbrück passed away after a long and arduous illness."

Professor of Psychology and Neuroscience, Ralph Adolphs, has joined the Biology Division while maintaining his initial appointment in the Humanities and Social Sciences Division. His laboratory investigates the neural underpinnings of human social behavior, pursuing questions such as: How do we recognize emotion from facial expressions? How do we make social judgments about other people? How do we look at people's faces (how do we move our eyes when looking at them)? How do we make decisions that are influenced by emotion? How do we remember emotional events in our lives? How do we make moral judgments about what is right and wrong? These questions are being addressed using a variety of techniques: (i) studying the impairments following specific brain damage in neurological patients; (ii) recording electrical activity from the brains of neurosurgical patients; (iii) probing the behavior of people with neuropsychiatric diseases such as autism and Williams syndrome; and, (iv) investigating behavior and brain activations in normal individuals with regard to social cognition.

Professor Dianne Newman has joined the Biology Division while maintaining her initial appointment in the Geological and Planetary Sciences Division. Bacteria have had and continue to have major impact on the chemistry of our environment. Dianne's approach includes genetic and molecular analysis of organisms that affect the production and dissolution of minerals. By understanding how existing organisms metabolize iron, for example, she hopes to better interpret the signatures that early life scrawled into the geologic record. Many of her favorite microbes develop into biofilms, with strata of individualized physiology. She and her group have developed novel ways of studying biofilms, structures crucial to microbial function and pathogenesis. By thinking about microbes from a geological perspective they have gained new insight into what might be the ancestral roles of microbial-produced antibiotics.

FERGUSON AWARD, 2005 - 2006

Meyerowitz **Bingol** **Schuman**

Dr. Baris Bingol is the winner of the Ferguson Award for the 2005-2006 academic year. This award goes to the student who is judged by the faculty to have produced the best Ph.D. thesis over the past year. Dr. Bingol performed his graduate studies in the laboratory of Professor Erin Schuman. His thesis work focused on the regulation of synaptic function by the ubiquitin proteasome pathway. He used a combination of biochemical, molecular biological and imaging techniques to demonstrate that a multi-protein cellular machine, the proteasome, moves into neuronal synapses when synapses are stimulated. The proteasome is responsible for the degradation of most cytoplasmic proteins.

PROFESSORIAL AWARDS, 2005 - 2006

Bren Professor of Psychology and Neuroscience, Ralph Adolphs, was awarded the 2005 Distinguished Investigator Award by the National Alliance or Research on Schizophrenia and Depression (NARSAD).

James G. Boswell Professor of Neuroscience, Richard A. Andersen, gave the Ian P. Howard Lecture in Vision Science at York University, Toronto, Canada, 2006.

Caltech President, Professor of Biology, Nobel Laureate, David Baltimore, was the 2006 President-Elect, of the American Association for the Advancement of Science.

James G. Boswell Professor of Neuroscience, Seymour Benzer, was awarded the Albany Medical Center Prize in Medicine and Biomedical Research, 2006.

Max Delbrück Professor of Biology, Pamela J. Bjorkman, in 2005, gave a Pfizer Lecture, Harvard University, Keynote Address, Antibody Engineering Conference, IBC's 16th Annual International Conference; and in 2006, was awarded the L'OREAL-UNESCO Women in Science North American Laureate, Paris, France.

Albert Billings Ruddock Professor of Biology, Marianne Bronner-Fraser, was elected to the Board of Trustees, Gordon Research Conferences and NIDCR Council; appointed to Scientific Advisory Board - Sontag Foundation (2006-present) and Scientific Advisory Board - March of Dimes Birth Defects Foundation (2005-present); elected to the Board of Directors, Society for Developmental Biology (2005-present); appointed to NHGRI Comparative Genome Evolution Review Committee and received a Javits Awards, NINDS.

Esther M. and Abe M. Zarem Professor of Bioengineering, **Michael Dickinson,** just completed an intensive eight-week laboratory and lecture course at the Marine Biological Lab at Woods Hole, MA. He and Sarah Bottjer of USC, served as the co-directors and instructors for the course that focuses on neural symptoms and behavior. This has been a three-year commitment and has one more year left in which to serve. He was invited to present the Clifford Ladd Prosser Lecture at the University of Illinois, Urbana-Champaign. Also, Joel Achenbach, who is a writer for the Washington Post, wrote an article on Michael's research and was published in National Geographic in June, 2006.

The Allen and Lenabelle Davis Professor of Biology, **Mary B. Kennedy,** received the 2006 Neuronal Plasticity prize of La Fondation IPSEN for research on "Synaptic protein complexes in neuronal plasticity." The prize was shared with Morgan Sheng and Eckart Gundelfinger.

Bing Professor of Behavioral Biology, Masakazu Konishi, was the recipient of The Gruber Prize in Neuroscience, from the Neuroscience Society, November, 2005; the Walter Heilgenberg Life Time Accomplishment Award, from the University of California, San Diego, April, 2006; and gave The Steve Kuffler Lectures, University of California, San Diego, May, 2006.

Lawrence A. Hanson Jr. Professor of Biology and Computation and Neural Systems, Gilles J. Laurent, gave the following lectures: David Bodian Lecture, Johns Hopkins University; Heller Lecture, Hebrew University, Jerusalem; and, the Ernest C. Watson Lecture, Caltech.

George W. Beadle Professor of Biology and Division Chair, Elliot Meyerowitz, gave Phi Beta Kappa Lectures at the College of Mary Washington, Bucknell University, Colorado State University, and Willamette University in January and March 2006. He was elected a member of the Council of the National Academy of Sciences, to serve from 2006 to 2009; and was given the Centennial Award of the Botanical Society of America in 2006.

Professor of Biology and Geobiology, Dianne K. Newman, was named Investigator of the Howard Hughes Medical Institute in 2005.

During this academic year, **Paul H. Patterson** was appointed to a named professorship: **Anne P. and Benjamin F. Biaggini Professor of Biological Sciences**; he also gave the Watson Lecture on May 17, 2006.

Howard and Gwen Laurie Smits Professor of Cell Biology, Alexander Varshavsky, has received the 2006 March of Dimes Prize in Developmental Biology from the March of Dimes Foundation; the 2006 Griffuel Prize in Cancer Research from the Association pour la Recherche sur le Cancer (France); and, the 2006 Gagna and Van Heck Prize from the Fonds National de la Recherche Scientifique (Belgium). In 2006, Varshavsky gave the opening lecture at the FASEB symposium on ubiquitin and cellular regulation.

PASSING

Zus Haagen-Smit, widow of Professor Arie Haagen-Smit, died at age 95 on February 7, 2006. An Oral History in the Caltech Archives records interviews with Zus in March, 2000; it is devoted mainly to Arie's career (1900-1977), especially his discoveries of the nature and sources of smog and his activities relative to air pollution. Zus herself had the equivalent of a Master's Degree in Physiological Botany from the University of Utrecht, and after their marriage she continued to be intellectually involved in "Haagie's" work. After periods in Indonesia and at Harvard, the Haagen-Smits came to Caltech in 1937, Haagie as a bio-organic chemist and member of the Biology faculty, and Zus, with her husband and children a congenial and contributing family, part of the Caltech and Pasadena communities for many years. An Arie J. Haagen-Smit Memorial Award has been given annually since Haagie's death to an undergrad in biology or chemistry, who has made recognized contributions to Caltech.

The Biology Division hosted the following lectures:

Norman Davidson Lecture
November 18, 2005
Richard Axel
Department of Biochemistry and Molecular Biophysics and Pathology
HHMI, Columbia University Medical Center
"A molecular logic of olfactory perception"

Kroc Lectures
September 22, 2005
Professor James Berger
Biochemistry and Molecular Biology
University of California, Berkeley
"Molecular mechanisms for regulating the initiation of DNA replication"

November 9, 2005
Randy Schekman
Howard Hughes Investigator and Professor of Cell and Developmental Biology
University of California, Berkeley
"Vesicular traffic: Mechanism and disease implications"

Weigle Lectures
May 16, 2006
Leonard P. Guarente
Novartis Professor of Biology
Department of Biology, MIT
"SIR2, calorie restriction and aging"

Wiersma Visiting Faculty
January 25, 2006
Nicholas C. Spitzer
Professor of Biology
Neurobiology and Computational Neurobiology, University of San Diego
"Activity-dependent transmitter specification and receptor matching"

March 8, 2006
Professor Kevan Martin
Institute of Neuroinformatics, University of Zurich, Switzerland
"A rough guide to the circuits of neocortex"

May 31, 2006
Ruth Lehmann
Julius Raynes Professor of Developmental Genetics
Department of Cell Biology (Skirball)
Skirball Institute Program of Developmental Genetics
New York University School of Medicine
"Genetic dissection of cell migration *in vivo*"

Photos from Biology Visiting Committee reception at the Ritz-Carlton, May 16, 2006, taken by Bob Paz

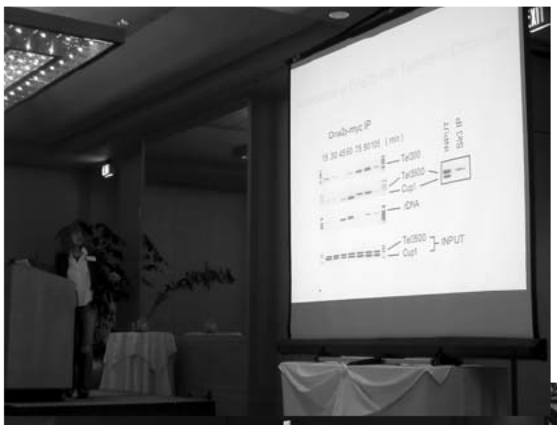








Above, photos from Biology Visiting Committee meeting at Caltech, May 16, 2006, taken by Tania Davis




Below, Photos from the Biology Division Retreat at Crowne Plaza Hotel, Redondo Beach, October 21 -23, 2005, taken by Patricia Mindorff



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Glenn C. Turner, *Ph.D.*
Phoebe Tzou, *Ph.D.*

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Summary: Our laboratory investigates the psychological and neural bases of emotional and social processing, using a number of different approaches. Some studies focus at the psychological level, using behavioral data from healthy people to make inferences about how emotion modulates memory, attention, or conscious awareness. Some of these measures are quite technically innovative and have been developed in our lab: for instance, measuring peoples' eye movements as they interact socially with one another.

A second approach uses neuroimaging to investigate the neural mechanisms behind emotional and social processing. The studies ask how the structure and function of the brain, in either health or disease, contributes to domains such as face processing or reward learning.

A third approach studies the performances, and the brains, of special populations. At Caltech, we have been recruiting people with agenesis of the corpus callosum to investigate the functional consequences of disruption in long-range connectivity. This work is spearheaded by visiting associate Lynn Paul, and involves collaborations with Michael Tyszka and Professor John Allman. In collaboration with Joe Piven at the University of North Carolina, we have also been studying people with autism, as well as their first-degree relatives (the parents). We are beginning to recruit people with autism locally at Caltech as well. At the University of Iowa, we have ongoing collaborations that involve neurological populations with focal brain lesions, and that involve neurosurgical populations in whom we can record intracranially.

The two primary aims of the research are to understand how emotion contributes to aspects of human social behavior and, in so doing, to help with the diagnosis and treatment of neurological and psychiatric illnesses that involve dysfunctional social behavior. A major focus has been on two brain structures: the amygdala, and the prefrontal cortex, both structures known to participate in emotion and social cognition. We are interested in how these structures modulate memory for emotional events, how they modulate allocation of attention to emotional stimuli, and how they guide decisions about options whose outcomes have emotional value. One particularly active area of development is to better understand how amygdala and prefrontal cortex interact, and how they interface with other brain structures that also participate in emotional processing. To this end, we are developing methods for analyzing the structural and functional connectivity of the human brain.

The studies involve Caltech collaborators from diverse backgrounds. Experiments on decision-making and reward-related learning involve faculty in the Division of the Humanities and Social Sciences who apply computational models to the analysis of human decision-making, such as Professors O'Doherty, Camerer, Rangel, and Bossaerts. Experiments on how emotion contributes to moral decisions even include faculty with backgrounds in philosophy, such as Professors Quartz and Woodward. In the Biology Division, we are collaborating with Professor John Allman on a variety of projects, and the investigations of the connectivity of the brain involve Michael Tyszka, Caltech's MR physicist.

1. Face-to-face eyetracking during social interactions

Michael Spezio, Sam Huang, Fulvia Castelli, Ralph Adolphs

The role of the human amygdala in real social interactions remains essentially unknown, although it is known from studies using artificial social stimuli (photographs of faces) to be critical for emotional processing and social cognition. We have recently studied a rare patient with complete amygdala lesions at Caltech, in a task in which she has to interact with another person. We monitored eye gaze during this experiment. The patient showed a severe reduction in direct eye contact in conversations with real people, together with an abnormal increase in gaze to the mouth. These findings are consistent with a hypothesized role for the amygdala in autism and open up new directions for quantifying social cognition and behavior in humans.

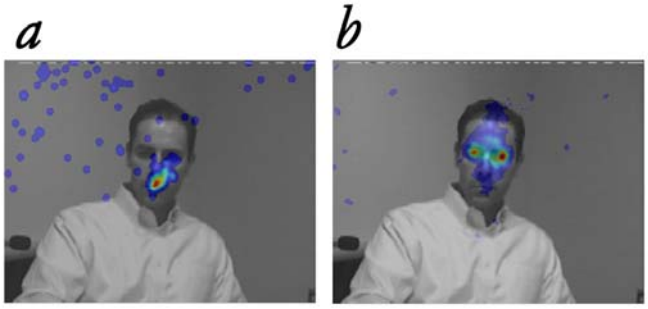

***Figure 1**: Damage to the amygdala impairs direct eye contact in conversations with real people. During a face to face conversation, a rare patient with complete and focal bilateral amygdala damage made almost no fixations to the eyes and almost exclusively fixated the mouth (a). In contrast, a group of age , gender , and IQ matched controls displayed normal fixations to the eyes during real conversation. Color indicates amount of gaze (Red: high; Blue: low).*

2. Facial categories do not attract attention

F. Moradi[1], R. Adolphs, S. Shimojo[2]

Recent studies suggest that some facial categories such as negative expressions that are not perceived nonetheless activate sub-cortical regions and amygdala. This unconscious pathway is suggested to direct attention toward ecologically significant categories so they are subsequently seen. However, it remains unclear whether it is stimulus features or emotion categories that are responsible for these effects. In order to investigate this issue, we examined whether or not categories guide attention in visual search among morphed faces.

We independently manipulated the distance of the stimuli in feature and in category space by positioning face morphs relative to category boundaries. Morphs equidistant in feature space are perceived as different if they span the category boundary, a phenomenon known as categorical perception. If facial categories rather than low-level image differences attract attention, then search time should depend more on the perceived difference between target and distracters than on how far apart they are in the morph continuum. Search displays comprised 4-16 faces, all but one of which were identical. We measured the reaction time that it took observers to report the location of the odd face.

We found that search was only slightly faster if the target differed from distracters in one categorical aspect (identity, expression, or gaze direction), and this contribution was small compared to the effect of distance in feature space. Furthermore, a rare patient with bilateral amygdala damage gave results essentially identical to normal observes (even for fear, which she cannot recognize from facial expressions). These findings argue that search for face categories is serial, and that deployment of focused attention in visual search does not depend on the amygdala, even for fear.

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3. Brain organization, social cognition, and emotional processing in agenesis of the corpus callosum

Lynn Paul, Warren Brown[1], Mike Tyszka, Matt Leonard, Nao Tsuchiya, Matt Bridgman[1], Dirk Neumann[2], Michael Spezio, Ralph Adolphs

Agenesis of the corpus callosum (AgCC) is a congenital condition in which all or part of the corpus callosum of a fetus does not develop. AgCC in individuals with intact general intelligence and no other major neuropathology is known as Primary AgCC, a rare condition studied primarily by Dr. Warren Brown's laboratory. Neuropsychological testing has revealed multiple deficits in this population, particularly in the areas of social and emotional cognition.

To date we have studied four such patients at Caltech. We have utilized well-established localizer tasks with fMRI to examine functional organization in AgCC, particularly visual field mapping, retinotopy, and tasks that localize fusiform gyrus, cingulate gyrus, and superior temporal sulcus. In a larger sample of eight adults with Primary AgCC, we have also measured eye tracking while the subjects look at emotional faces, complex scenes, and in visual search tasks. Preliminary eye-tracking results in three AgCC subjects, indicating unusual patterns of eye movements when viewing photographs of faces, were presented at the International Neuropsychological Society conference in February, 2006.

Ultimately, correlation of brain activation findings and behavioral outcomes may provide critical insight about the functional mechanisms underlying the social-emotional deficits in AgCC. In turn, this understanding may also inform the role of interhemispheric transfer and corpus callosum development in behavioral syndromes that involve similar deficits, such as autism spectrum disorders.

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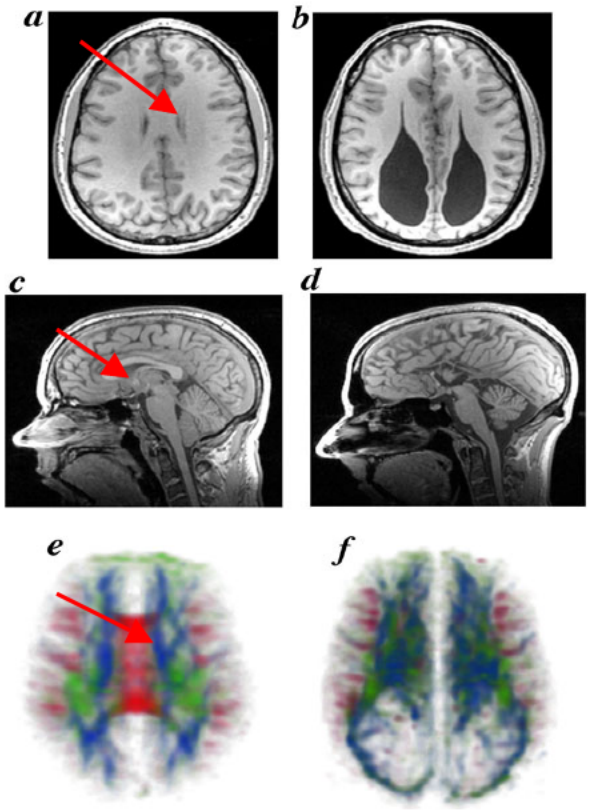

Figure 2: Structural features of the live brains of individuals with agenesis of the corpus callosum. Structural magnetic resonance images are shown for a healthy brain (***a,c***) and a brain with ACC (***b,d***). White interior regions are fibers ("white matter"), the convoluted rim of the interior brain are regions containing brain cells ("grey matter"), and the brain is enclosed by the skull and scalp (thin white outer shell). Panels ***a,b*** show horizontal sections at the level of the white line that is indicated in image ***d*** for orientation. Panels ***e,f*** show a view of the entire brain seen from above (front of the brain is towards the top of the image) in which the orientation of connections between different brain regions have been color-coded. Connections going top-bottom are blue, front-back are green, and ones that go from left to right are red.

The red arrow indicates the location of the corpus callosum in the healthy brain (compare corresponding location in the ACC brain to see its absence

4. Structural and functional integration in callosal agenesis

J. Michael Tyszka, Lynn Paul, Dirk Neumann[1], Matthew Leonard, Matthew Bridgman[2], Scott E. Fraser[3], Ralph Adolphs

Agenesis of the corpus callosum (AgCC) is a relatively rare human developmental abnormality in which the corpus callosum is either completely or partially absent. Many adult AgCC subjects have normal IQs and are asymptomatic in routine neurological or psychiatric examinations. As the rate of commissurotomies declines, individuals with AgCC are becoming our primary source of information about callosal transfer in the human brain. AgCC is particularly interesting to those studying network plasticity and compensation since these subjects do not exhibit classical disconnection syndromes. The specific focus of this project is to develop a more complete understanding of long-range structural and functional integration and the compensatory networks associated with AgCC using state-of-the-art non-invasive imaging and advanced statistical modeling. To date we have studied five adult AgCC subjects using a battery of magnetic resonance imaging techniques including BOLD fMRI of coordinated bimanual motor tasks, somatosensory stimulation, high angular resolution diffusion imaging (HARDI) and time-of-flight angiography. Probabilistic fiber tracking (PFT) is now a routine tool for analysis of HARDI data and infers structural connectivity by modeling restricted anisotropic water diffusion within tissues in a non-deterministic framework. We are currently developing methods for integrating PFT with BOLD fMRI in order to characterize the remodeled motor system in AgCC from both the structural and functional standpoints. This project represents a multi-disciplinary, multi-center collaboration between the Divisions of Biology, the Humanities and Social Sciences, Cognitive Neural Systems and the Fuller Graduate School using the advanced resources of the Caltech Brain Imaging Center.

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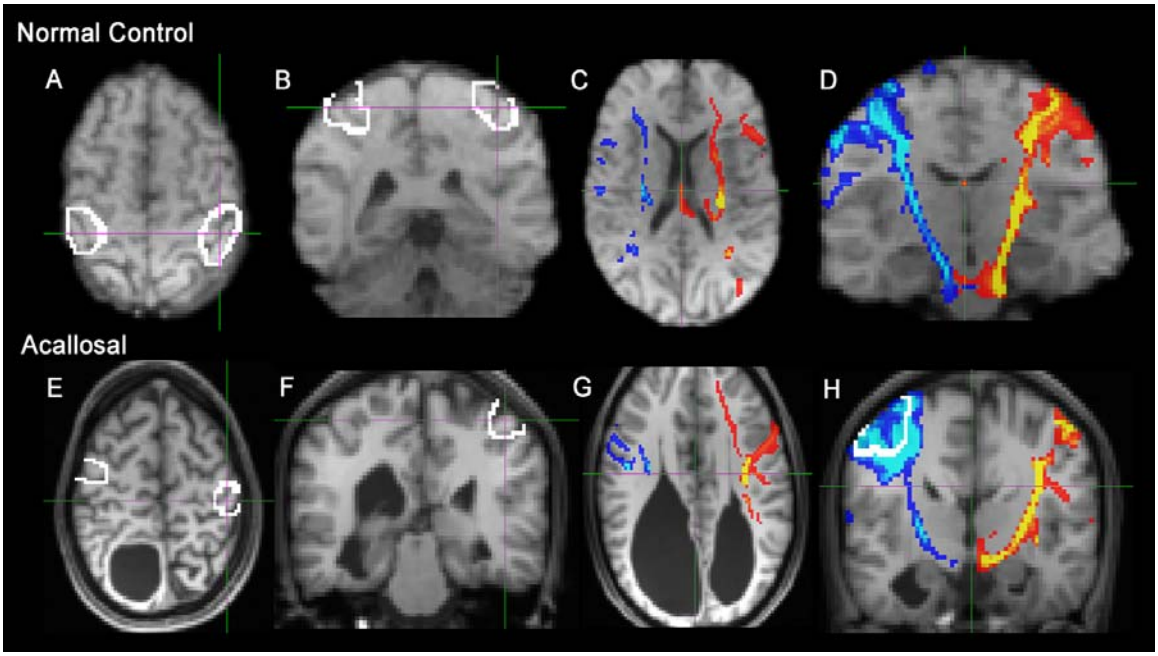

***Figure 3**: Integrated probabilistic fiber tracking and BOLD fMRI localization in a normal control and AgCC subject. The left and right primary motor areas were localized using a coordinated finger tapping task (A,B,E,F). Probabistic fiber tracking results are overlaid on coregistered high resolution structural MRI (C,D,G,H). A total of 5000 fibers were traced for each seed voxel within the motor areas. Exploration of the use of such data as a constraint or prior for connectivity modeling is a key element of this project. Note that in this subject, the occipital horns of the lateral ventricles were significantly enlarged (colpocephaly, visible in axial section in E,G) as were other CSF spaces.*

5. Autobiographical memories of moral events

Jessica Edwards, Ralph Adolphs

Morality, moral rules and moral choices have provided rich fodder for philosophers, psychologists and others. Moral judgments play a key role in many of our daily social interactions. However, the neuroscience behind moral decision-making is not yet well understood. This project investigates how we recollect moral autobiographical events and how we make moral judgments about them. Using a cued recall protocol in which subjects are given specific words ("tell me about the time you felt most guilty"), we have collected nearly 800 moral memories from 100 local, healthy, adult subjects (ages 40-60). In addition, these subjects have been broadly characterized, allowing us to examine the possible associations between neuropsychological profiles (such as personality traits) and specific types of memories. Currently we are developing a searchable database of the moral memories that they generated, including independent ratings of the moral choices in each memory. This database will facilitate the selection of moral memories to use subsequently as stimuli in a variety of projects investigating the role of moral events in memory, decision-making, and other aspects of social cognition.

6. Deficit in parafoveal processing of negative emotions following bilateral amygdala damage

Naotsugu Tsuchiya, Ralph Adolphs

For more than a decade, we have been characterizing a rare patient, SM, who has fairly circumscribed and selective damage to bilateral amygdala. A recent study of SM's gaze revealed a highly abnormal overt attention to faces: she showed a dramatically reduced fixation to the eye region of faces (Adolphs *et. al.* (2005), *Nature*). Because the eye region contains the most useful information for discriminating fear from other emotional expressions (Smith *et. al.* (2005), *Psychological Science*), SM's deficit in recognizing fearful expression may be due to her failure to fixate the eye region of faces. Indeed, when instructed to look at the eyes, SM was able to recognize fear. This still leaves us with a puzzle: why does SM fail to look at the eye region of faces spontaneously?

One possibility is that SM fails to process salient eye regions when they are located parafoveally – such normally salient stimuli may not attract her attention, resulting in less frequent fixations onto the eyes. To test this hypothesis, we characterized her ability to discriminate briefly presented facial expressions at parafoveal locations. In each block of ~100 trials, one of six basic emotional expressions (happy, sad, fearful, angry, disgusted, and surprised) served as a target. In each trial, two faces (one emotional, and the other neutral; both of the same identity) were briefly presented (40 msec) slightly lateralized on both sides of fixation. Subjects were asked to judge which side showed the target emotional face as

accurately and rapidly as possible. In half of the trials, both faces were inverted (upside-down).

SM gave a performance close to chance in discrimination of every negative emotion (sad, fearful, angry and disgusted), but performed entirely normally for happy and surprise. SM also showed intact performance in control experiments, discriminating unpleasant natural scenes (mutilated bodies, crying babies, etc.) from landscape pictures under the identical presentation conditions (40 msec). Another bilateral amygdala patient (AS) showed the same pattern of specific impairment.

Our results implicate the amygdala in encoding the saliency of negative facial expressions, which may in turn trigger eye movements towards such faces normally.

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7. Abnormal processing and eye gaze to facial features in autism

Michael Spezio, Ralph Adolphs, Robert Hurley, Joseph Piven

Altered visual exploration of faces likely contributes to social cognition deficits seen in autism. To investigate the relationship between face gaze and social cognition in autism, we measured both face gaze and how facial regions were actually used during emotion judgments from faces. For this, we presented subjects with "bubbles"—stimuli in which only small portions of faces are revealed (Fig. 4). One can then correlate the features of faces revealed in these sparse stimuli with the performance accuracy of subjects in discriminating the emotion shown, to derive the potent features that subjects actually use in the discrimination task.

Compared to IQ-matched healthy controls, nine high-functioning adults with autism (HFA group) failed to make use of information from the eye region of faces, instead relying primarily on information from the mouth (Fig. 5). Face gaze accounted for the increased reliance on the mouth, and partially accounted for the deficit in using information from the eyes. These findings provide a novel quantitative assessment of how people with autism utilize information in faces when making social judgments.

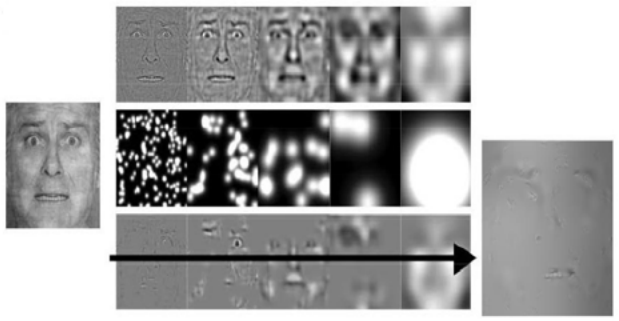

Figure 4. *Construction of the "bubbles" stimuli. One of four emotional facial expressions (e.g., image at far left), with equal energy across all spatial frequencies (hence the somewhat grainy quality) was decomposed into five octaves of spatial frequency that were randomly sampled to generate a sparsely revealed face stimulus (far right).*

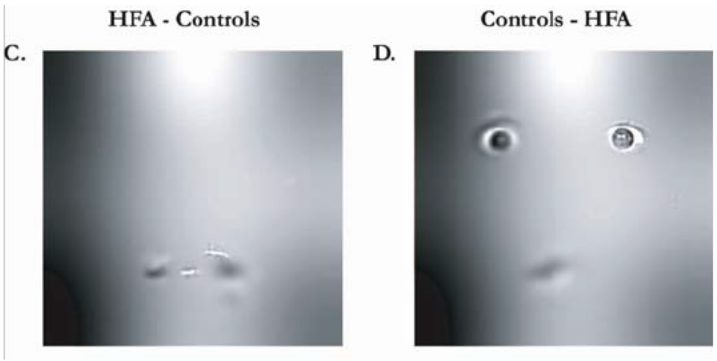

Figure 5: *Classification images showing the difference between how high functioning adults with autism (HFA) and matched controls actually use visual information in the face while making social judgments. The HFA group relied much more on high frequency visual information in the mouth (C), while the controls relied more on the eyes (D).*

8. Emotional reactions to facial features

Dirk Neumann[1], Frédéric Gosselin[2], Ralph Adolphs

Fearful faces produce an immediate emotional reaction in viewers. While the features responsible for the accurate identification of facial expressions have been studied, comparatively little is known about the features subtending autonomic responses to facial expressions. To identify the regions of a face that influence emotional arousal, we sampled images of faces with randomly located Gaussian apertures (Gosselin and Schyns, 2001). The pictures of four individuals (two females) from the Ekman face set expressing fear and neutrality were normalized for the position of their main facial features and their energy spectra were equalized. We asked seven participants to passively view the images while we continuously measured their galvanic skin response (GSR). For each trial we computed the amplitude of the GSR, heart rate, the respiration rate, and estimated the degree to

which the visual information at each location of the image influences the response. The resulting maps of canonical correlation coefficients were smoothed with a Gaussian kernel and thresholded at an overall significance level of p <0.05. We found a significant modulation of autonomic responses when the eyes or parts of the mouth were revealed. Autonomic emotional reactions to faces appear to be driven most potently by the eyes, consistent with the hypothesis that such emotional responses also contribute to our overt classification of the emotions shown in facial expressions.

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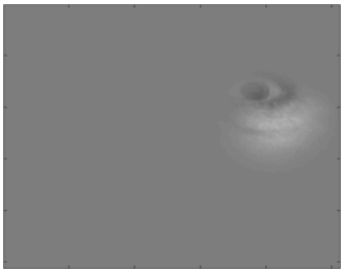

Figure 6*. Region that significantly influences autonomic responses when fearful facial expressions are shown.*

9. Probabilistic tractography of the connectivity of the human brain

Dirk Neumann[*]*, J. Michael Tyszka, Ralph Adolphs*

Two challenges in tracking axonal connections in the brain are to track fibers that are small compared to the imaging resolution, and to track through voxels that contain multiple and crossing fibers. Diffusion-weighted imaging with high angular resolution, together with probabilistic models of apparent diffusion, can help surmount these difficulties. However, the relationship between the uncertainty in the apparent diffusion measurement and the uncertainty of the fiber track estimates remains poorly understood.

Here we studied the reliability of a probabilistic fiber-tracking (PFT) algorithm by looking at the agreement of fiber estimates for interhemispheric connections seeded in contralateral voxels. We used a statistical measure, Kullback Leibler (KL) divergence, that does not make any assumption about the shape of the probability distribution to quantify the difference in the probability distribution describing paths from a seed regions in the left hemisphere to the right hemisphere and that from the right to the left hemisphere. The KL divergence is the average amount of information, expressed in number of bits that are wasted when encoding the events from one distribution with the optimal code from the other distribution. Thus, if the two distributions were identical, they would have a KL divergence of zero, and the larger the KL divergence, the larger the non-overlap between the two distributions (i.e., the lower the reliability of the fiber tracking algorithm).

Scans were acquired with a horizontal bore 3T Siemens system using a diffusion-weighted EPI sequence (64x64x34 matrix, 3 mm resolution, 72 directions). The orientation density distribution was estimated using the FMRIB's diffusion toolbox. Seed regions were automatically labeled using the FreeSurfer cortical parcellation package. Custom software was used for the probabilistic sampling and Runge Kutta line integration.

Initial probabilistic fiber tracking estimates were quite reliable. The improved PFT algorithm (PFT+) that uses regularization of the orientation distribution function (trilinear interpolation, Runge Kutta integration) resulted in a significant improvement in reliability (p<.001). The KL divergence provides a quantitative approach to study the reliability of probabilistic fiber tracking results.

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10. Dissociating top-down and bottom-up visual attention in autism

Dirk Neumann[1]*, Michael Spezio, Joe Piven*[2]*, Ralph Adolphs*

People with autism are impaired in their social behavior, including their eye contact with others, but the processes that underlie this impairment remain elusive. We combined high-resolution eye tracking with computational modeling in a group of ten high-functioning individuals with autism to address this issue. The group fixated the location of the mouth in facial expressions more than did matched controls, even when the mouth was not shown, even in faces that were inverted, and most noticeably at latencies of 200-400 ms. Comparisons with a computational model of visual saliency argue that the abnormal bias for fixating the mouth in autism is not driven by an exaggerated sensitivity to the bottom-up saliency of the features, but rather by an abnormal top-down strategy for allocating visual attention.

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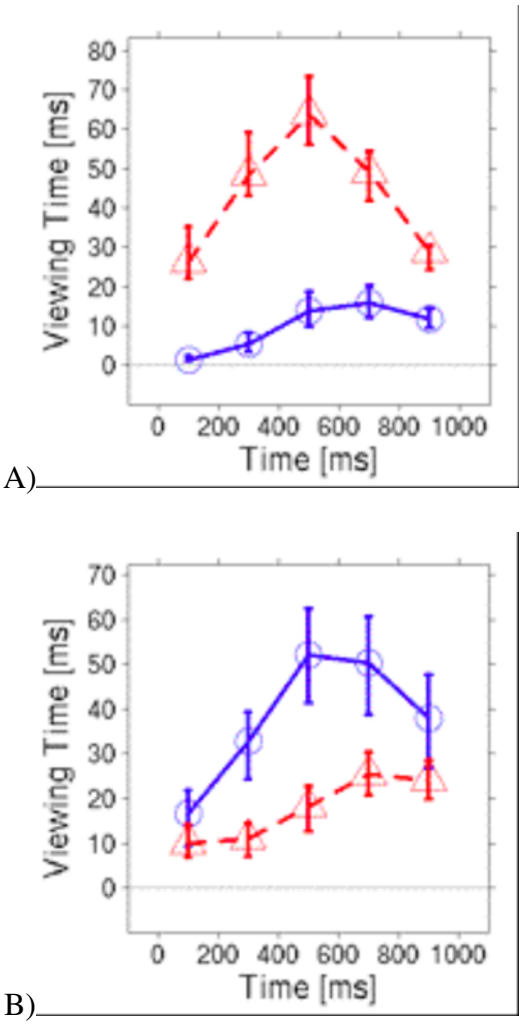

Figure 7. Fixation times when looking at the stimuli for A) the *mouth* and B) the *eyes.* Viewing times of the autism group is red, the control group is shown in blue.

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Summary: We are mainly concerned with brain evolution as revealed through the comparative study of brain structure and with the neural mechanisms of economic and social decision-making. These two interests come together in our investigation of the Von Economo (spindle) neurons of anterior cingulate and fronto-insular cortex. These neurons are present only in humans and apes and are much more abundant in humans than in apes; they thus represent a recent development in hominoid evolution. The Von Economo cells emerge mainly after birth and are 30% more abundant in the right hemisphere. We think that the Von Economo neurons are part of the circuitry responsible for rapid intuitive choice in complex social situations.

Another facet of our laboratory research is the incorporation of neuroimaging modalities, particularly MRI, into the analysis of comparative brain anatomy. We are investigating the structure of the brain in another highly social mammal, the African elephant, using MRI. And we are currently working with a new variant of MRI, called diffusion tensor imaging, which can be used to characterize the orientation and coherence of white matter tracts in the brain. In order to quantitatively validate this emerging technology, we are comparing measurements of fiber anisotropy from DTI scans with measurements of fiber coherence as measured from histological preparations performed on the same specimens.

11. A comparative study of the hypothalamus in apes and humans

John Allman, D. L. Lei[1], J.R. Korenberg[2], P. Hof[3], A.Y. Hakeem, J.A. Kaufman, K.F. Manaye[1]

The paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus have long been known to maintain autonomic functions including fluid and electrolyte homeostasis, but recent research has also shown that these nuclei are involved in central processes such as social affiliation and emotional cognition that are frequently disturbed during aging and in neuropsychiatric disorders. The SON and PVN consist of arginine vasopressin (AVP) - and oxytocin (OT)-synthesizing neurons that send projections to the neurohypophysis. The PVN also projects to other brain areas including cortex. In the present study, we quantified Nissl-stained neurons using unbiased stereology in the PVN and SON in human (n=8), gorilla (n=1), and chimpanzee (n=3). In the PVN, the mean total number of neurons was significantly higher in humans (300,000) compared to the chimpanzees (110,000) and gorilla (134,000). In contrast, no significant differences were present in the total numbers of neurons in SON among the three species. The results indicate that SON, a region of the hypothalamus involved in social bonding and nurturing, contains the same number of neurons in humans compared to apes, while the human PVN, which is involved learning and higher cortical functions, including cognition, has almost three times as many neurons as are present in the apes. We have previously reported that there was a 50% reduction in the number of PVN neurons in patients of major depression and bipolar disorders. The greatly expanded population of PVN neurons in humans is the product of recent evolution and may be particularly vulnerable to pathology. By understanding variations in the neuronal circuitry related to social bonding, emotions, and cognition in human and apes, we may be better able to interpret pathological variation in humans.

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12. Von Economo neurons are selectively vulnerable in agenesis of the corpus callosum

Jason A. Kaufman, Lynn K. Paul[1,2], Kebreten F. Manaye[3], Julie R. Korenber4[5], Patrick R. Hof[5], John M. Allman

Agenesis of the corpus callosum (AgCC) can have significant effects on social and emotional behaviors, including alexithymia (the inability to express one's emotional state), difficulty intuiting the emotional states of others, and deficits in self- and social-awareness that can impair such capacities as humor, comprehension of non-literal or affective language, and social judgment. One component of the neural network that supports social cognition which may be damaged in AgCC is the von Economo neurons (VENs), which are large spindle-shaped neurons localized to two brain regions that are known to be involved in social and emotional cognition: anterior cingulate cortex (ACC) and fronto-insular cortex (FI). VENs appear late in development (emerging mainly postnatally), are a recent phylogenetic specialization and, as a consequence may be particularly vulnerable to certain neuropathologic conditions.

To test the hypothesis that VENs are selectively vulnerable in AgCC, we used stereology to obtain unbiased estimates of total neuron number and VEN number in postmortem brain specimens of two normal adult controls and two adults with isolated callosal dysgenesis. In both ACC and FI, the partial agenesis case displayed approximately half as many VENs as in the two normal controls. In the complete agenesis case the VENs were almost entirely absent. Most importantly, the ratio of

total neurons to VENs increases dramatically in callosal agenesis (Figure 1). These results indicate that VENs are selectively vulnerable in AgCC. Additionally, complete AgCC appears to be more detrimental to the VEN population than partial AgCC, suggesting that even a limited corpus callosum may provide a sustaining connection for the VEN population. We conclude that abnormal brain development can have a particularly harmful effect on VENs, and that their vulnerability could help to explain some social and emotional deficits that are seen in a variety of brain disorders.

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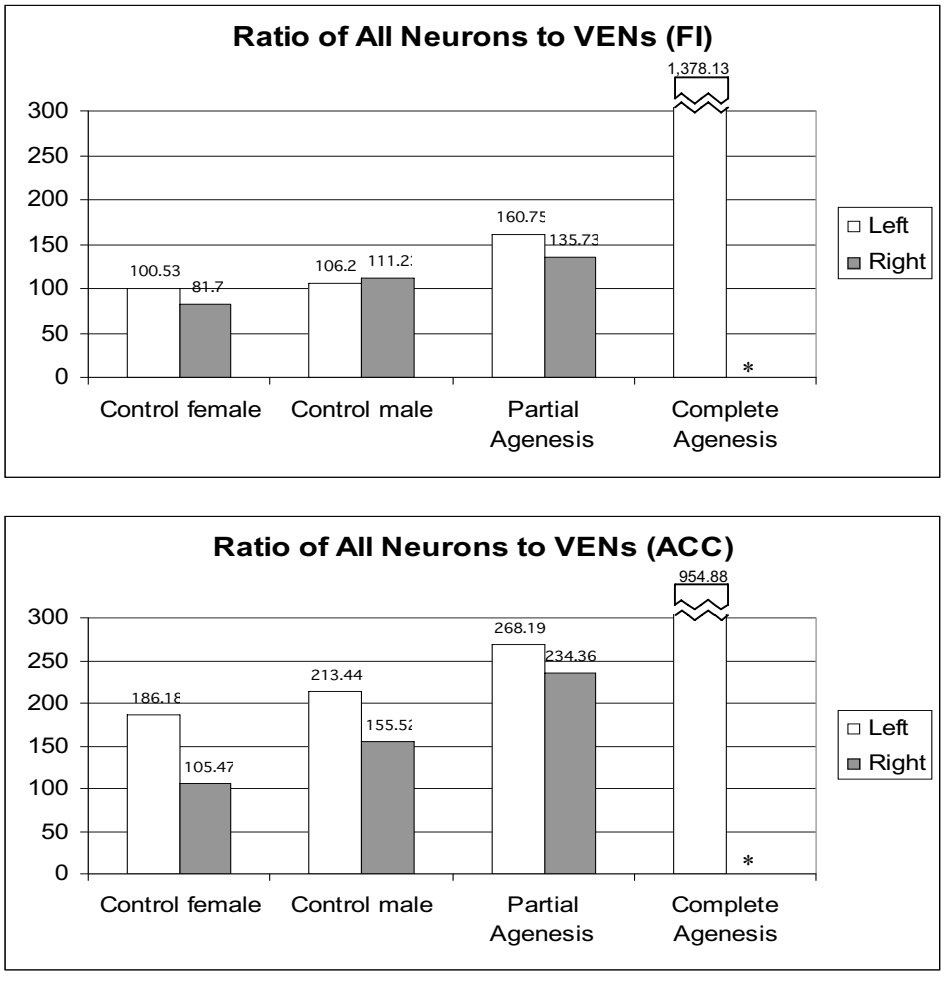

Figure 1. Ratio of total neuron number to VEN number in FI (top) and ACC (bottom) obtained from stereology. In the partial AgCC case, the ratio of all neurons to VENs is slightly higher than in controls, while in the case of complete AgCC the ratio is dramatically higher, indicating that VENs are selectively vulnerable in callosal agenesis. *Left hemisphere of the complete AgCC case was not suitable for stereology.

13. White matter pathways in the brain of a gorilla revealed by high-field diffusion MRI

Jason A. Kaufman, J. Michael Tyszka, Patrick R. Hof[1], John M. Allman

Cerebral white matter consists of organized bundles of axons that provide long-distance connections between different cortical regions, and between cortex and sub-cortical grey matter structures. Traditional methods for tracing long-distance fiber tracts require terminal injection procedures or focal lesion and degeneration studies, both of which are impractical and unethical for controlled experimentation in humans and apes. Diffusion-weighted magnetic resonance imaging (DW-MRI) is a new, non-invasive means of obtaining data on brain connectivity. Within any particular voxel, DW-MRI provides a quantitative measure of the coherence of axon fibers – a property termed fractional anisotropy – as well as the principal orientation of those fibers in three-dimensional space. Tract-tracing algorithms use these diffusion data to track fiber pathways from voxel to voxel.

We scanned a formalin-fixed gorilla brain using a 9.4 Tesla MR imaging system to obtain ultra-high-resolution structural images (250 μm isotropic voxel size) and high angular-resolution diffusion images (72 diffusion directions; 1mm isotropic voxel size). With the high-field imaging system, we were able to achieve anatomical scans with 4-6 times the resolution of most structural MRI experiments, and our diffusion-weighted scans have at least twice the resolution of even the most sophisticated diffusion-spectrum MRI studies (Figure 1). Visualization of the principal diffusion direction (Figure 1, right) reveals distinct white matter fiber tracts that correspond to known pathways, including the corona radiata, cingulum, internal capsule, and longitudinal fasciculi, as well as interhemispheric callosal pathways. In the near future we hope to apply probabilistic fiber tractography algorithms that will allow us to trace these and other major fiber pathways throughout the brain. We believe that by applying this technology in a comparative context across primate species, quantitative comparisons of the strength and distribution of cortical connections may reveal distinct specializations related to the evolution of the human brain.

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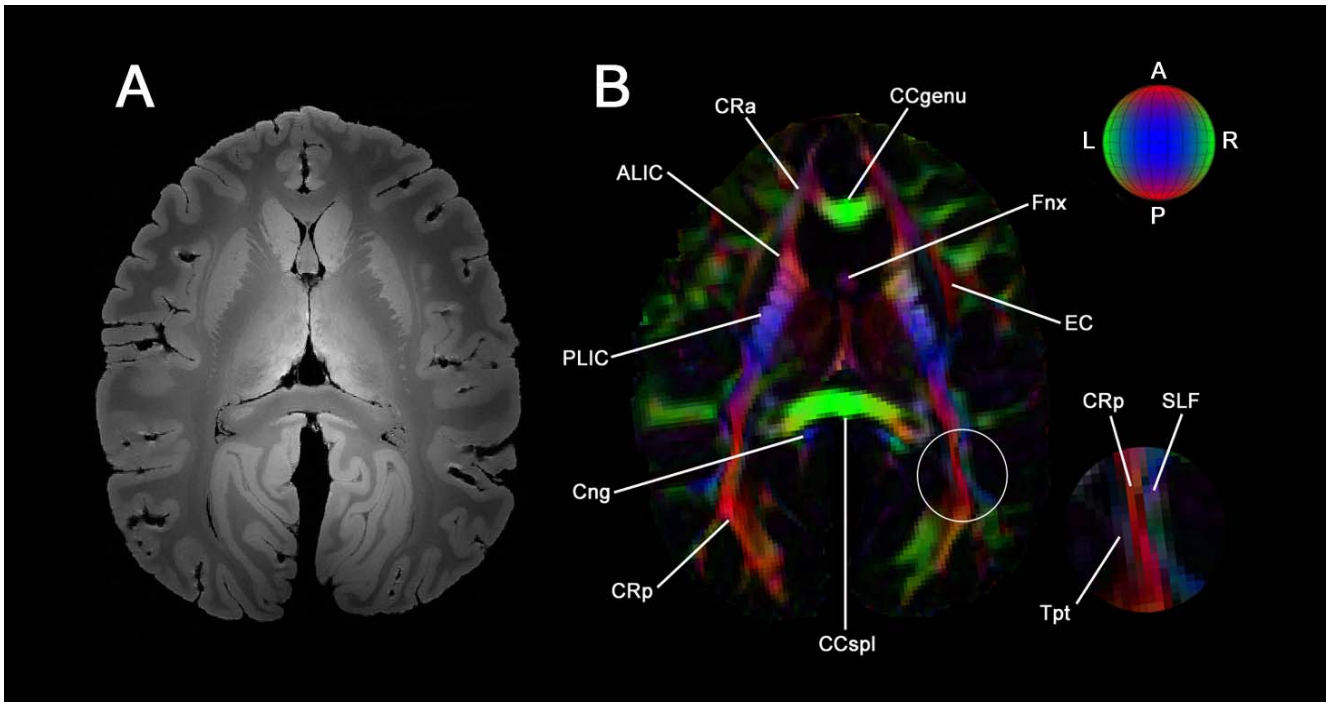

Figure 1. **A**: Axial slice through the ultra-high-resolution structural MR image. The structural image volume was acquired in 3D using a FLASH sequence with a final resolution of 250 μm isotropic. **B**: Visualization of the principal diffusion direction in which each voxel is assigned a color based on the orientation of fibers within the voxel (see color legend at top right). Approximately the same level as in **A**. The diffusion image volume was acquired using a 3D PGE sequence with a final resolution of 1mm isotropic. Abbreviations: ALIC, anterior limb of the internal capsule; PLIC, posterior limb of the internal capsule; EC, external capsule; CCgenu, corpus callosum genu; CCspl, corpus callosum splenium; Cng, cingulum bundle; CRa, anterior part of the corona radiata; CRp, posterior part of the corona radiata; Fnx, fornix; SLF, superior longitudinal fasciculus; Tpt, tapetum.

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Neural mechanisms for visual-motor integration, spatial perception and motion perception

Summary: While the concept of artificial intelligence has received a great deal of attention in the popular press, the actual determination of the neural basis of intelligence and behavior has proven to be a very difficult problem for neuroscientists. Our behaviors are dictated by our intentions, but we have only recently begun to understand how the brain forms intentions to act. The posterior parietal cortex is situated between the sensory and the movement regions of the cerebral cortex and serves as a bridge from sensation to action. We have found that an anatomical map of intentions exists within this area, with one part devoted to planning eye movements and another part to planning arm movements. The action plans in the arm movement area exist in a cognitive form, specifying the goal of the intended movement rather than particular signals to various muscle groups.

Neuroprosthetics. One project in the lab is to develop a cognitive-based neural prosthesis for paralyzed patients. This prosthetic system is designed to record the electrical activity of nerve cells in the posterior parietal cortex of paralyzed patients, interpret the patients' intentions from these neural signals using computer algorithms, and convert the "decoded" intentions into electrical control signals to operate external devices such as a robot arm, autonomous vehicle or a computer.

Recent attempts to develop neural prosthetics by other labs have focused on decoding intended hand trajectories from motor cortical neurons. We have concentrated on higher-level signals related to the goals of movements. Using healthy monkeys with implanted arrays of electrodes we recorded neural activity related to the intended goals of the animals and used this signal to position cursors on a computer screen without the animals emitting any behaviors. Their performance in this task improved over a period of weeks. Expected value signals related to fluid preference, or the expected magnitude or probability of reward were also decoded simultaneously with the intended goal. For neural prosthetic applications, the goal signals can be used to operate computers, robots and vehicles, while the expected value signals can be used to continuously monitor a paralyzed patient's preferences and motivation.

Movable probes. In collaboration with Joel Burdick's laboratory at Caltech, we have developed a system that can autonomously position recording electrodes to isolate and maintain optimal quality extracellular recordings. It consists of a novel motorized microdrive and control algorithm. The microdrive uses very small piezoelectric actuators that can position electrodes with micron accuracy over a 5 mm operating range. The autonomous positioning algorithm is designed to detect, align, and cluster action potentials, and then command the microdrive to move the electrodes to optimize the recorded signals. This system has been shown to autonomously isolate single-unit activity in monkey cortex. In collaboration with Yu-Chong Tai's lab and the Burdick lab, we are now developing an even more compact system that uses electrolysis-based actuators designed to independently move a large number of electrodes in a chronically implanted array of electrodes.

Coordinate frames. Our laboratory also examines the coordinate frames of spatial maps in cortical areas of the parietal cortex coding movement intentions. Recently, we have discovered that plans to reach are coded in the coordinates of the eye. This is particularly interesting finding because it means the reach plan at this stage is still rather primitive, coding the plan in a visual coordinate frame rather than the fine details of torques and forces for making the movement. We have also discovered that when the animal plans a limb movement to a sound, this movement is still coded in the coordinates of the eye. This finding indicates that vision predominates in terms of spatial programming of movements in primates.

We have also been examining the coordinate frame for coordinated movements of the hand and eyes. In the dorsal premotor cortex we find a novel, "relative" coordinate frame is used for hand-eye coordination. Neurons in this cortical area encode the position of the eye to the target, the position of the hand to the target, and the relative position of the hand to the eye. A similar relative

coding may be used for other tasks that involve the movements of multiple body parts such as bimanual movements.

Motion perception. Another major effort of our lab is to examine the neural basis of motion perception. One series of experiments is determining how optic flow signals and efference copy signals regarding eye movements are combined in order to perceive the direction of heading during self-motion. These experiments are helping us understand how we navigate as we move through the world. A second line of investigation asks how motion information is used to construct the three-dimensional shape of objects. We asked monkeys to tell us which way they perceived an ambiguous object rotating. We found an area of the brain where the neural activity changed according to what the monkey perceived, even though he was always seeing the same stimulus. In other experiments, we have been examining how we rotate mental images of objects in our minds, so called mental rotation. In the posterior parietal cortex we find that these rotations are made in a retinal coordinate frame, and not an object-based coordinate frame, and the mental image of the object rotates through this retinotopic map.

Local field potentials. The cortical local field potential (LFP) is a summation signal of excitatory and inhibitory dendritic potentials that has recently become of increasing interest. We report that LFP signals in the saccade and reach regions provide information about the direction of planned movements, as well as the state of the animal; e.g., baseline, planning a saccade, planning a reach, executing a saccade, or executing a reach. This new evidence provides further support for a role of the parietal cortex in movement planning. It also shows that LFPs can be used for neural prosthetics application. Since LFP recordings from implanted arrays of electrodes are more robust and do not degrade as much with time compared to single-cell recordings, this application is of enormous practical importance.

fMRI in monkeys. We have successfully performed functional magnetic resonance imaging (fMRI) experiments in awake, behaving monkeys. This development is important since this type of experiment is done routinely in humans and monitors the changes in blood flow during different cognitive and motor tasks. However, a direct correlation of brain activity with blood flow cannot be achieved in humans, but can in monkeys. Thus, the correlation of cellular recording and functional MRI activation in monkeys will provide us with a better understanding of the many experiments currently being performed in humans. A 4.7 Tesla vertical magnet for monkey imaging has recently been installed in the new Imaging Center in the Broad building. We are using this magnet, combined with neural recordings, to examine the correlation between neural activity and fMRI signals.

14. Dynamics of context modulations in the "reach" network

M. Brozovic, A. Gail, R.A. Andersen

The anti-reach task is well suited for studying how context influences the dynamics of sensorimotor transformations. In the anti-reach task the spatial sensory input is kept the same, while a colored context cue instructs the animal to reach either toward (pro-reach) or away (anti-reach) from the visual target. Our neural network study is based on the experimental evidence that shows that the neurons in the parietal reach region (PRR) predominantly encode the position of the motor goal as opposed to the position of the visual cue. Furthermore, the motor goal representation in PRR is delayed by ~60 ms in anti- compared to pro-reaches, implying the need for an additional step of cortical processing. The time dynamics of the contextual motor planning provides evidence for a recurrent network.

In neural network simulations we find that a sensorimotor network for reaches (visual areas -> parietal reach region (PRR) -> downstream areas) works mainly in a feed-forward fashion for pro-reaches. The movement goal corresponds to the visual cue location and there is no need for a transformation. However, for anti-reaches the neural network strongly depends on feed back projections. Anatomically this feedback may be derived either from within PRR or from areas downstream of PRR such as dorsal premotor cortex or dorsal prefrontal cortex. For anti-reaches, these pathways bring in the context information needed to update the motor plan in PRR.

15. Selection of targets and active disregard of irrelevant information in monkey LIP and SEF

M. Campos, B. Breznen, R.A. Andersen

Using asynchronous presentations of a target and distracter in a saccade task, we believe we have uncovered two important components of the neural mechanism of target selection, including some evidence for the active disregard of irrelevant information. In a study of the neural representation of objects in oculomotor structures prior to learning an object-based task, we used a paradigm in which objects were presented, but were irrelevant for task completion. We recorded single-unit activity from cortical areas SEF and LIP in two monkeys during this variant on the memory-guided saccade task, in which a distracter stimulus (one of two green polygonal objects) was presented prior to the cue stimulus (small while dot), which was briefly flashed. The distracter persisted until just before the signal for the monkey to saccade to the remembered location of the spatial cue. The location of the target and distracter were each chosen randomly from a set 42 peripheral locations covering the visual field out to 17 deg in all directions. Neither of the monkeys had previous exposure to any tasks in which the distracter stimuli (objects) were relevant. Many LIP neurons (26%, 31/120), showed spatially tuned responses to both the distracter and cue presentations, though the distracter response was transient (100%, 31/31), and the cue response was maintained (84%, 26/31), throughout the memory period. Thus, in agreement with many published findings, LIP responds transiently to all flashed stimuli, but

selectively maintains representations of intended eye movement targets. Many SEF neurons respond in a still more selective manner, most (24%, 40/165), responding selectively only to the cue, and not responding to the distracter. Surprisingly, some (9%, 15/165), SEF neurons responded only to the distracter, and were silent during the presentation of the cue. These SEF responses could be thought to label the task relevance of the target and distracter stimuli. Another class of SEF neurons responded to both the cue and distracter, but both of these responses tended to be transient, perhaps marking the passage of time in an event-based manner (Campos *et al*, 2005). Taken together, we find that, first, frontal cortex labels relevant and irrelevant stimuli, and second, parietal cortex selectively maintains the spatial representations required for planning behavior. These are important components of target selection when various stimuli are presented at different times.

16. Posterior parietal cortex encodes non-spatial decision-making for effector selection

H. Cui, R.A. Andersen

The posterior parietal cortex (PPC) of rhesus monkeys has been found to encode impending decisions regarding the spatial goals of movements. The present study addresses the encoding of non-spatial decisions between two effectors instead of two locations in the space. In other words, the decision concerns '*how'* rather than '*where.'* We examined PPC activity while monkeys chose to acquire a target either by saccading or reaching in the absence of direct instructions specifying the effectors. The monkey was seated in front of a board with an array of buttons, each containing a red and a green LED. At the beginning of the trial, both the green and red LEDs in the central button were turned on and the monkey was required to fixate and touch it. Then both the red and green LEDs in a peripheral button were turn on simultaneously, and the monkey was required to continue fixating and touching the central fixation spot until it disappears (GO signal). After 600 ms of cue duration, the green (red) LED was turn off and only the red (green) remained on in 25% of trials, instructing a saccade (reach) after delay. In the remaining 50% of the trials, both peripheral LEDs extinguished, and the monkey could decide to either shift gaze to the location of the peripheral target while continuing to touch the center spot, or keep fixation of the center spot but move the arm to reach to the target. In these free choice trials, the monkey was rewarded only if his choice matched the computer's choice and computer biased its choice against the monkey's choice sequence. This competitive algorithm provided an effective method to balance the monkey's bias for saccade/reach selection. We recorded 33 lateral intraparietal (LIP) cells and 68 parietal reach region (PRR) cells from two macaque monkeys, and 16 LIP cells (48%) and 35 PRR cells (51%) exhibited significant effector-specific selectivity for the monkeys decisions, with LIP selective for decisions to saccade and PRR selective for decisions to reach. For those effector-selective cells, the activity in saccade and reach choice trials began to separate at variable times, spanning from 20 ms after cue offset to movement onset. Our results suggest that the PPC also encodes non-spatial decisions regarding effectors choice, and decision making appears to be a progressive and involve multiple and at least partially non-overlapping circuits in the PPC.

17. Local field potentials represent context-specific movement goals in the posterior parietal cortex of monkeys

A. Gail, R.A. Andersen

We performed a memory-guided anti-reach experiment to analyze the dynamics of sensorimotor transformations in the posterior parietal cortex. We determined the relative timing of neural activity related to visual sensory processing and reach movement planning in the parietal reach region (PRR) of monkeys. In an instructed-delay, center-out reach task the monkey had either to reach to a memorized peripheral target position (PRO-reach) or to a diametrically opposed position (ANTI) while keeping central ocular fixation. PRO- and ANTI-trials were pseudo-randomly interleaved and indicated to the monkey from the beginning of the trial by a color cue. We analyzed PRR single-unit spiking activity and local field potentials (LFP) with respect to spatio-temporal response selectivity. We have shown previously that the ensemble spiking activity encoded the sensory information on the cue position weakly and only during a short period of cue visibility. From the end of the visual cue presentation and during the memory period the representation of the reach goal was increasingly prevalent in the ensemble spiking activity. This means that PRR encodes context-specific sensorimotor transformations. We now show that in PRR LFPs in the frequency range above 15 Hz, but not below, are spatially tuned, and that their tuning also reliably represents the reach goal during the memory period, regardless of whether the monkeys are planning a PRO or ANTI reach. In contrast to the spike activity the visual cue location is not represented in the ensemble LFP spectral amplitudes, even during cue visibility. This is mostly likely due to the small fraction of single PRR neurons that were tuned for the visual cue during the cue period in our data, and the integrative effect of LFP over a large number of neurons. LFPs in PRR exclusively encode the specific information on the movement plan, regardless of the context. Together with their superior properties for stable long-term recordings, this confirms the suitability of LFPs for the use in neuroprosthetic devices.

18. fMRI during goal-directed movement planning in normal and spinal cord injured subjects

H.K. Glidden, N. Yozbatiran[*]*, D.S. Rizzuto, S.C. Cramer*[*]*, R.A. Andersen*

It has been proposed that the posterior parietal cortex (PPC), dorsal premotor cortex (PMd), and supplementary motor area (SMA) may be good locations for the implantation of a cortical brain-machine interface. This study evaluates the feasibility of using functional magnetic resonance imagined (fMRI) and imagined movements to localize potential neuroprosthetic implant sites in paralyzed patients and aid in neurosurgical planning. Spinal cord injured (SCI) patients planned and executed imagined limb movements towards remembered

visual locations in a modified memory reach task. Across five SCI subjects scanned thus far (time since injury: 1-10.5 years, mean-3.85 years; age: 22-60 years, mean =36 years; lesion level: C5-T5/T6, three subjects: C6/C7), we find that many of the same brain areas are activated compared to normal controls. With this limited sample size, we also notice a shift in the left hemispheric-laterality for imagined right leg movements in normal subjects towards a right hemispheric-laterality in SCI subjects, particularly in PMd and SMA. Additionally, PPC activity appears to be more bilateral in SCI subjects than in normal subjects performing the same task. Since imagined leg and arm movement planning activate highly overlapping areas with real reach planning in normal subjects, it is possible that in these SCI patients a more normal pattern of brain activation is maintained by use of the upper limbs, exercise and rehabilitation therapy, etc. Another concomitant study of the same SCI patients addresses changes in brain activation patterns during attempted knee movement before and after intensive exercise therapy. These studies address the question of whether there are different patterns of reorganization in PPC, PMd and SMA compared to primary motor and somatosensory areas. We continue to recruit and scan normal and SCI subjects and to characterize and correlate activity patterns with lesion level, time since injury, age, and other clinical factors.

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19. Local field potentials as a control signal for brain machine interfaces

E.J. Hwang, S. Musallam, H. Scherberger, R.A. Andersen

In the posterior parietal reach region (PRR) of macaque monkeys, both the spike firing rates and the local field potentials (LFPs) in the ~20 Hz band are directionally tuned during the memory period of the memory guided reach task. Furthermore, LFPs at the ~10 Hz band are modulated by behavioral state, i.e., the LFP power at ~10 Hz becomes weaker during the memory period compared to the movement or fixation periods. The directional tuning and behavioral state dependent modulation suggest that LFPs can be used as a robust control signal for the control of a brain machine interface (BMI). To test this idea, we examined LFPs recorded from chronically implanted electrode arrays in PRR and compared the tuning properties from the arrays with those from acute single-electrode recordings. Consistent with the results from the acute recordings, LFPs from the chronic arrays were directionally tuned mainly at the ~20 Hz band during the memory period and LFP power at ~10 Hz was strongly modulated by behavioral state. More interestingly, the tuning strength of the LFPs at ~20 Hz was not correlated with the tuning strength of the spike firing rates from the same electrode channel, suggesting that the LFP signal can provide direction information even when little or no tuning exists in the firing rate of neurons on the same channel. This independent directional tuning of LFPs is an appealing feature for a control signal for BMIs since the isolation of neurons from an array is not guaranteed. We are currently testing the effect of electrode location and electrode impedance on the tuning strength of the LFPs in order to optimize the recording of LFPs for use in a BMI.

20. fMRI of reward context modulation during motor response preparation

A. Iyer, I. Kagan, A. Lindner, R.A. Andersen

Effects of reward and punishment have been extensively investigated in humans with functional imaging, mostly focusing on subcortical and frontal cortical structures implicated in the immediate processing of task contingencies. These studies are paralleled by electrophysiological investigations in monkeys, mostly demonstrating consistent analogies between reward processing in the two species. However, recent recordings in monkeys also showed dependence of activity of single neurons on reward contingencies in many cortical areas that have not been directly implicated in the processing of reward. For example, in delayed response tasks, monkey parietal areas PRR and LIP exhibit firing rate modulation based on the expectancy for amount and probability of reward. The few studies that addressed preparatory activity in humans concentrated mostly on prefrontal and premotor areas.

Here, we used event-related fMRI to investigate the effect of expected reward or punishment, contingent on the task performance, in human cortical areas recruited in spatial memory and motor planning tasks. We added a reward/punishment context to a variant of the delayed response task in which subjects had to remember locations of spatial cues and use this information for subsequent motor responses, using trackball to perform sequences of "virtual" reaches with a cursor. Subjects were given monetary reward or punishment based on their performance, on a trial-by-trial basis. The current reward/punishment context for the trial was indicated shortly before the presentation of the spatial cues. The memory load and the response complexity were demanding so that performance level would not exceed a certain level, usually <75% correct. We used different combinations of reward/punishment context to dissociate between effects of expected value and motivation.

Our preliminary results suggest that besides structures involved in the processing of expected and received reward in humans, several other cortical regions exhibit modulation with the reward/punishment context in the early phase of the delay period, i.e., shortly after the presentation of the context and spatial cues. In particular, areas along intraparietal sulcus, in middle frontal gyrus (at the junction of superior frontal sulcus and precentral sulcus), and precuneus were more activated in trials where possible gain or loss was high, as compared to trials where anticipated gain or loss was small or null. Further experiments will elucidate potential reward modulation during later phases of delay period activity.

21. Event-related fMRI in alert behaving monkeys and humans during visually-guided and memory saccades

I. Kagan, A. Iyer, A. Lindner, S. Wagner[*]*, R.A. Andersen*

Delayed (memory) response paradigms have been used extensively in monkey electrophysiology and more recently in human functional imaging studies in order to dissociate visual and motor responses, and to investigate mechanisms of working memory and movement planning. However, the exact relationship between these studies is not clear due to differences in methods and time-scales. Here we provide a direct comparison between the two species with the same tasks and techniques, using a high-field 4.7T vertical MRI scanner for monkeys and a 3T scanner for humans. Eye movements, reward, timing and other behavioral information (e.g., body or head motion in monkeys) were recorded while subjects made visually guided and memory saccades to visible or remembered cues. The performance of the task and the behavioral feedback was controlled in real-time. Additionally it was necessary to detect and remove motion-affected periods from the monkey data.

Advancing previous monkey fMRI studies that used block design, we applied event-related analysis of BOLD signal time-courses to delineate responses from different epochs within the task sequence – fixation, cue, delay period, saccade execution, reward expectation and acquisition. In the first experiment we compared responses during visually- and memory-guided saccades to extract "cognitive" signals related to spatial working memory and motor preparation. In monkeys, prefrontal and parietal areas, in particular discrete bilateral regions in *arcuate sulcus*, principal sulcus, intraparietal sulcus, and superior temporal sulcus, exhibited spatially specific, contralateral cue and memory/preparation activity. The event-related time-courses from these areas revealed cognitive demand-related differential signals seen as a separate peak or elevated activity in the middle and late memory period, distinguishable from the early cue response. Consistent activation patterns were found in putative functional human homologs, although exact shape and contribution of these signals varied between subjects in both species. In control experiments we varied the duration of the memory period and the relevance of the cue for subsequent action to further separate sensory cue response from the memory/preparatory processing. Our results demonstrate that dynamics of "higher-order" cognitive signals can be detected in monkeys using event-related fMRI, making it a powerful link between human imaging and monkey electrophysiology.

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22. Dynamical gain fields in MSTd

B. Lee, M. Brozovic, B. Pesaran, R.A. Andersen

Neurons in the dorsal aspect of the medial superior temporal area (MSTd) respond to the focus of expansion (FOE) of the visual image and demonstrate eye position gain fields. Previous studies characterized MSTd gain fields as planar, planar component, no planar component, and no gain. Here, we report that MSTd gain fields change their gain-type based upon the retinal input. In the fixed gaze (Fixed) condition, the monkeys fixated at five eye positions, six degrees apart, along the preferred direction of pursuit. Focus of expansion (FOE) tuning was determined at each of the five eye positions. In this condition, the input signals to MSTd were the expanding visual stimulus and eye position signals. In a simulated pursuit (Sim) condition, the monkey also fixated at five eye positions while the same stimulus used in the Fixed condition was dragged across the screen. In this condition, in addition to the expanding visual stimulus and eye position signals, there was also laminar motion of the retinal input from the simulated pursuit stimulus. We found that 33/73 neurons did not change their gain field-types from Fixed to Sim. This included the neurons that did not have gain-fields in both Fixed and Sim (19/73). 40/73 neurons changed their type of gain fields between Fixed and Sim. Through decode analysis (Fisher linear discriminant), we found that eye position was closely tied to a small subpopulation of (14/73) neurons which we call "eye cells." These neurons did not change the type of their gain-fields between the Fixed and Sim conditions. The decode of eye position from the neurons which changed their gain was significantly lower. For comparison, the decode of the focus of expansion was the same for neurons which did and did not change their gain fields. We conclude that the dynamical gain fields associated with MSTd are due to a combination of eye position and the retinal signal input and this mechanism may play a role in self-motion processing.

23. fMRI correlates of human spatial working memory and movement planning in the posterior parietal cortex

A. Lindner, A. Iyer, I. Kagan, R.A. Andersen

The posterior parietal cortex (PPC) plays a critical role in motor planning a notion that stems mainly from electrophysiological studies in non-human primates engaging delayed response tasks. Such tasks temporally separate task instructions and motor responses from the intervening memory- and motor planning- phase, permitting the delineation of neuronal contributions to each of these processes. Recently, similar experimental designs have been applied during fMRI in humans, mainly focusing on a putative role of PPC in retrospective visual memory and/or attention rather than on its role in motor planning. Here we tease apart event-related fMRI-responses associated with retrospective visual memory and prospective motor planning during the delay period.

Ten human subjects performed delayed response tasks in which sequences of virtual "reaches" had to be executed with a finger-controlled trackball. In the 'delayed reach task' (i) movements were instructed by cues that were presented before the delay period, whereas the 'non-match to sample task' (ii) these cues indicated undesired target locations. The actual motor response in the latter task was defined by sample cues that were presented after the delay period: movements should be performed only to new target locations. Thus, reaches could either be prepared in advance (task i) or be avoided (task ii), while visual memory load (two or four spatial cues) was kept constant across conditions.

Significant delay period activity for both tasks was found in PPC, premotor cortex (PM) and dorsolateral prefrontal cortex (BA46). This delay period activity was significantly modulated by the memory and/or planning load in PPC and PM. Activity related to motor planning was mainly confined to the PPC, where fMRI-responses during the delay differed between the two task conditions: fMRI-responses in the non-match to sample task were significantly larger than in the delayed reach task. In control experiments (n=3), in which the motor response could no longer be predicted by the initial cues (match to sample task), PPC activity was reduced as compared to the delayed reach and non-match to sample tasks, again supporting the role of PPC in motor planning.

This pattern of activity in PPC cannot be explained solely by retrospective visual memory, which was identical across conditions. It rather indicates an additional involvement of PPC in the planning of upcoming movements while coding for both types of targets relevant for action – those to be acquired and those to be avoided.

24. Online directional control signals in posterior parietal cortex

G. Mulliken, S. Musallam, R.A. Andersen

Psychophysical and clinical studies in humans point to a role for PPC in rapid online updating and correction of movements. To explore the neural representation of online control signals in primate PPC, we analyzed the correlations of single-neuron activity with four plausible sensorimotor control angles (SCAs) during a visually guided joystick task, including: 1) angle to target in eye coordinates (EYE-to-TGT); 2) angle to cursor in eye coordinates (EYE-to-CUR); 3) angle to target in cursor coordinates (CUR-to-TGT); and, 4) heading angle of cursor (CUR HEAD).

To enforce curvature and ensure online control of trajectories, we trained one monkey to use a joystick to guide a cursor on a computer screen around an obstacle and to a target. The monkey initiated a trial by moving a white cursor (0.9 deg) into a central green target circle (4.4 deg) and fixating a red concentric circle (1.6 deg). After 350 ms, a blue obstacle circle (10.0 deg) appeared and the target jumped simultaneously to one of eight random peripheral locations (14.7 deg). The obstacle was aligned and equidistant to the initial and end target positions. The monkey guided the cursor around the obstacle and into the peripheral target. Once the cursor was within 2.2 deg of the target center for 350 ms, he was rewarded with a drop of juice. If the cursor intersected the obstacle or central fixation was broken, the trial was aborted.

We constructed space-time receptive fields (STRFs) using reverse correlation to assess SCA tuning for 50 neurons. STRFs typically exhibited unimodal SCA tuning and were largely separable in the SCA-time plane. We classified cells according to which SCA was best encoded. Variance in STRF time slices was used to assess SCA tuning strength as a function of lag time. The time slice with maximal variance was defined as the optimal lag time (OLT) for a particular SCA. The SCA whose maximal variance was significantly larger than all others was defined as the best encoded SCA (bootstrap, $p<0.05$; t-test). We found that 11 neurons (22%) best encoded EYE-to-TGT, 17 neurons (34%) best encoded EYE-to-CUR (OLT=44ms), 18 neurons (36%) best encoded CUR-to-TGT (OLT=-7ms), and four neurons (8%) best encoded CUR HEAD (OLT=30ms). These results show that during online control of a cursor PPC dynamically encodes both the state of the cursor and the target in eye and cursor-centered coordinates.

25. Neurons in the posterior parietal cortex code the amplitude of an impending reach

S. Musallam, R.A. Andersen

When planning a reach, the direction and the amplitude of the reach need to be specified. Surprisingly, a study of motor cortex found that the variation in firing rate due to the amplitude of the movement usually occurs during the movement time and not before it (Fu *et al*., 1993). Recently, premovement reach amplitude information was found in premotor cortex (Messier and Kalaska 2000). Given its role in high-level motor planning, we examined whether amplitude information is also found in the posterior parietal cortex (PPC). Two monkeys were trained to perform memory reaches to targets located at various distances from the fixation point in a 2D plane. Using acute recordings, 63 neurons were recorded from the parietal reach region (PRR) of Monkey C. Of these, 53 neurons (84%) responded to the memory reach task and how many were direction selective. 45/53 (84%) were modulated by the target distance. 22/45 (49%) neurons had distance tuning in the cue period, 31/45 (69%) in the memory period, and 21/45 (47%) in the motor period. 32/45 (71%) neurons increased their firing rate in response to an increase in the amplitude of the reach in the neuron's preferred direction while 13/45 (29%) neurons decreased their firing rate. In the second animal (monkey S), neurons were recorded from an array of 32 electrodes implanted along the medial bank of the intraparietal sulcus. From these 32 channels, 21 single units were recorded of which 15 were tuned to the memory reach task. Only 3/15 (20%) of these neurons were modulated by amplitude during the cue period, all 15 were modulated in the motor period and 7/15 (46%) were modulated in the memory period. All the neurons that encoded the amplitude of the reach were directionally tuned. In the same session, we ran a brain-control experiment using the memory period activity of the seven memory-period tuned neurons to decode the location of eight possible targets at four different directions and two different amplitudes in each direction. We were able to decode the targets with a success rate of 73.7% (chance =12.5%). We conclude that amplitude is encoded in the reach plan in PPC and that goals located anywhere in a 2D plane can be decoded from this region.

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26. Stimulus- and percept-dependent biases of V1 neuronal activity for ambiguous three-dimensional objects

Z. Nadasdy, B. Pesaran, R.A. Andersen

Ambiguous visual objects enable the study of perception under constant sensory input conditions. Two rhesus monkeys were trained to report the percept of ambiguous structure-from-motion objects interspersed with unambiguous objects while single-unit responses and LFP were recorded from the superficial layer of V1 neurons. In order to elucidate the effects of previous trials on the neuronal responses and perceptual decisions, we varied the duration of inter-trial-intervals (ITIs) between two time scales, a short (ITI<1s) and a long (ITI>2s) time scale. We estimated the probability with which the firing rate and the LFP spectrum of V1 neurons allow an ideal observer to predict the monkey's perceptual choice. Neuronal responses to zero-disparity (ambiguous) objects were sorted according to the perceptual choices, the type of object in the preceding trial, and the inter-trial interval. As many as 40% of the neurons showed a significant perceptual bias which was dependent on the object presented in the previous trial and on the duration of ITI. Specifically, for the majority of biased neurons the firing rate increased during an ambiguous trial following a trial rotating in the neuron's preferred direction after long ITI if the monkey reported motion consistent with the neuron's preferred direction. In contrast, firing rate was reduced in trials preceded by objects rotating in the anti-preferred direction following long ITIs when the monkey reported anti-preferred direction motion. Interestingly, this effect was reversed under the ITI<1s condition. In both cases the monkey's choices were consistent with the late neuronal responses; namely, the percept "froze" following ITI>1s and "switched" following ITI<1s. Early responses correlated more with the previous trial while late responses showed more correlation with the percept. This suggests that perceiving ambiguous objects in one way or another is not merely a by-chance decision but rather is biased by the primary visual cortex in response to previous exposures.

27. Computing time from space in the parietal reach region

E.B. Torres, R.A. Andersen

We address a new question - is there a difference in PRR cells responses between a motion that requires thinking to solve it, and one that is performed in automatic mode? In complete darkness while fixating straight ahead two rhesus monkeys performed interleaved delayed center-out (DCO) reaches (automatic task) with DCO reaches around obstacles (naïve task) to the same peripheral targets. We concurrently recorded the responses from PRR neurons with the arm motions. During the 1000 ms of memory period 126/144 neurons from both animals showed significant differences (two-way ANOVA, $p = 0.01$) in mean firing rates and tuning properties when the postural (and) hand paths changed from straight to curved. In addition, during the obstacle condition 117/144 neurons continued to change such response properties after the spatial path had been resolved but the speed was being planned. Analysis of behavioral data from this period revealed that (1) the arm posture was repositioned prior to the movement in anticipation to the upcoming curved path, and (2) during the first impulse of the motion there was fine-tuning of the distance traveled to the first velocity peak while for each target the time to reach this peak was held constant. We asked if the first significant changes were due to the transformation of the hand goal into a postural plan and the second significant changes were due to the distance-based speed planning. To this end we added an experiment where the initial change in posture was enforced so that the postural paths changed relative to those starting from the normal posture but where the speed profiles did not change. The experimental sequence was DCO-normal, DCO-ob, DCO-normal, DCO-enforced, and DCO-normal. In 33/35 PRR cells there were significant changes in gain when the upcoming postural paths changed both due to the spontaneous postural repositioning in OB-avoidance and to the enforced initial postural change. In 29/35 cells, there were significant changes in gain during the OB-condition for distance-based temporal planning. In contrast for the enforced-posture case 31/35 cells had no significant changes when there were no required changes in speed profiles. These results suggest involvement of PRR cells in path planning at the postural level and distance-based temporal planning reflected later in the first impulse of the motion.

28. Floating microelectrode arrays for chronic implantation

C. Ustun, S. Musallam, M.J. Bak, P.R. Troyk, D. Rizzuto, C. Pang, Y.C. Tai, R.A Andersen

A vital component of many types of Brain-Machine Interfaces (BMI) is a chronically implantable array that can record from neurons that lie near the surface of the brain or deep within the convoluted structures of the cortex. Development of such an array will be important for the development of BMIs and, in particular, neural prosthetics for paralyzed patients. Here we report on metal-based and silicon-based arrays that satisfy the above requirements.

We developed a microwire-based array consisting of up to 18 electrodes attached perpendicularly onto a thin ceramic substrate. The array is wired to a connector via a set of fine gold wires enabling it to "float" in the cortex, thus preventing the relative motion of the brain with respect to the skull from producing damage to the neural tissue. Arrays with electrodes insulated with Parylene and made from Pt/Ir with lengths of either 1 mm or 8 mm were implanted in rats. In two rats with 1 mm long probes, single-unit activity was observed in 4/8 and 10/16 channels with additional channels showing multi-unit activity. In one rat with an 8 mm array extending into the caudate, 10/16 channels showed either single- or multi-unit activity. We also observe local field potentials in 15/16 channels in the deep array.

We also present arrays manufactured using silicon-chip technology. An advantage of this approach is that signal processing and wireless communication electronics can be integrated directly into the array design, potentially leading to significant decreases in cost and increases in reliability. Our manufacturing technique allows probes to have arbitrary numbers of shanks, shank lengths (up to 10 mm) and contact impedances. The first

generation array was made with seven shanks spaced 400 μm apart. Each shank has four contacts spaced 125 μm apart and has an impedance ranging from 500 kOhms to 1.5 M Ohms. The width and thicknesses of the shanks at the base are 150 μm each and taper toward a point. The array is designed to penetrate the rat dura (and the primate pia) without breaking. We have tested this array for mechanical integrity in the rat and we plan to implant rats and monkeys in the upcoming months.

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Summary: There are currently three major areas of investigation in this laboratory: the development of neural stem cells; the functional neuroanatomy of emotional behaviors in mice; and the functional neuroanatomy of innate behaviors in *Drosophila*.

Neural stem cell biology
Stem cells are multipotent, self-renewing progenitor cells. In the central nervous system (CNS), neurons, astrocytes and oligodendrocytes the three major cell classes of the CNS are thought to be derived from self-renewing stem cells. In contradistinction to this prevailing view, our recent studies in the spinal cord suggest that following the period of neurogenesis the majority of progenitor cells do not self-renew, but rather lose neurogenic capacity and become restricted to a glial fate. Current work is aimed at identifying genes that control this irreversible switch from neurogenesis to gliogenesis. An understanding of this mechanism might eventually permit controlled reversion of adult glial cells to a stem cell-like state, which could be useful for therapeutic purposes.

Neural circuitry of behavior
We are developing and applying molecular biological tools to map and manipulate the neural circuitry underlying emotional behaviors in mice. These studies focus on two distinct but related states: pain and fear. In the former case, we have identified a novel family of G protein-coupled receptors (GPCRs) for neuropeptides, called Mrgs, which are specifically expressed in restricted subsets of primary nociceptive sensory neurons. Using homologous recombination in embryonic stem cells, we have marked the neurons that express different Mrgs with genetically-encoded axonal tracers. Remarkably, different Mrg-expressing neurons project to different, and highly specific, peripheral target tissues. We are now engaged in genetic inactivation or killing of these neurons, as well as genetic activation, to understand their function. We are also tracing the higher-order projections of these neurons into the brain, to determine the point at which these novel and distinct sensory circuits converge.

In a separate project, we have identified genes that are expressed in subpopulations of neurons in the central nucleus of the amygdala, which is thought to be a major output structure that co-ordinates different aspects of learned fear responses. Using the promoters of these genes, we are generating mice in which these neurons can be reversibly silenced, in collaboration with Henry Lester's laboratory. The role of these neurons, and the circuits in which they participate, in mediating responses to conditioned and unconditioned fearful stimuli, can then be assessed.

In parallel with these studies in mice, we are engaged in conceptually similar experiments in the fruitfly, *Drosophila melanogaster*. Our goal is to identify simple and robust innate behaviors, and then perform unbiased "anatomical" and genetic screens to map the neuronal circuits and identify the genes that control these behaviors. This dual approach will provide an opportunity to integrate molecular genetic and circuit-level approaches to understanding how genes influence behavior. The "anatomical" screen exploits the availability of "enhancer trap" lines, in which the yeast transactivator protein GAL4 is expressed in specific subsets of neurons, and a conditional (temperature-sensitive) neuronal silencer gene (*shiberets*) that prevents synaptic transmission. Currently, we have developed assays for an innate avoidance response triggered by an odorant mixture released from traumatized flies, as well as for arousal intensity and hedonic valence, two important axes underlying emotional states in humans.

29. Identification of genes associated with the neuron to glia developmental transition

Ben Deneen, David J. Anderson

Development of the central nervous system (CNS) is dependent upon the appropriate spatial and temporal generation of the three main neural cell types: neurons, oligodendrocytes, and astrocytes. While the spatial dorsal-ventral combinatorial code responsible for the differing neuronal cell types generated in the ventricular zone has been deciphered, the mechanisms governing the temporal switch from neuronal to glial generation remain elusive. The goal of my study is to identify genes that control the transition from neurogenesis to gliogenesis.

As a model for this developmental transition we chose to utilize Olig-expressing cells, because the Olig genes are specifically expressed in the pMN domain of the developing spinal cord during both neurogenic and gliogenic phases of development. In this study we utilized an Olig2/GFP transgenic mouse in order to prospectively isolate Olig2-expressing cells via FACS analysis from

embryonic spinal cord across this developmental interval (E9.5 though E12.5). Using these methods of cell isolation, we have been able collect sufficient numbers of cells from each timepoint to generate cRNA probes to hybridize to Affymetrix microarrays.

To test the validity of the candidate genes identified in our microarray experiments, we performed *in situ* hybridization on numerous genes. In particular, we analyzed the expression of several members of a family of transcription factors, the NF1 genes, which are upregulated across this time interval. This family of genes demonstrates specific spatial expression throughout the ventricular zone at E12.5, suggesting that these genes could play a role in the neuron to glia fate switch. Preliminary gain- and loss-of-function studies in the chick neural tube suggest that these genes may indeed function to promote the onset of gliogenesis. Currently, we are examining the effects all members of the NF1 family, individually or in combination, have on the onset of gliogenesis through gain-and loss-of-function analysis in the chick neural tube.

30. Molecular and cellular manipulations of Mrgprd+ nociceptive neurons

Liching Lo, Mark J. Zylka, David J. Anderson

Nociceptors are a large population of afferents that have a high threshold for activation and responds preferentially to intense, noxious stimuli. *Mrgprd* is expressed in a subset of pain-sensing nociceptive neurons (~75% of the IB4+ neurons). Mrgprd+ free nerve endings terminate exclusively in the epidermis and do not innervate any other cutaneous structures, such as sweat glands, Merkel cells, or Meissner corpuscles. Mrgprd+ fibers do not innervate any other tissues in the body. In the dorsal horn, Mrgprd+ and CGRP+ neurons project to distinct lamina layers. Because of the exclusive projection and innervation, it is important to know how the brain processes these specific afferent inputs and how this dedicated projection compares to other projections with different target specificities. Tracing the second or third order projections should provide us some answers. To achieve that, we have used gene targeting in ES cells to generate strains of mice in which the coding region of Mrgprd was replaced with Cre recombinase or the tetracycline-dependent transcriptional activator (rtTA). To circumvent the position effect seen in many transgenic mice, the tetO-WGA (a lectin, wheat germ agglutinin) or tetO-DTA (diphtheria toxin A) was put downstream of rtTA in the same orientation in the same targeting cassette. For transneuronal tracing analysis of circuits, the MrgD-Cre mice were crossed with ZW mice (gift from Allan Basbaum) in which excision of the lacZ gene by Cre-mediated recombination initiates expression of the WGA. For cell ablation study, administration of doxycycline should induce the expression of diphtheria toxin, resulting in the cell autonomous kill of only the Mrgprd neurons. We have generated and are in the process of analyzing these mice.

31. Molecular specification of motor neuron progenitor cell fate in the CNS

Agnes Lukaszewicz

In the battle against Amyotrophic Lateral Sclerosis, replacing the degenerating neurons appears to be one of the most promising treatments. For this reason, an improved basic knowledge of motor neurons should aid in the search for a cure. A first step toward this mission would be to better understand the molecular mechanisms of motoneuron fate specification during development. To do so, one of the most relevant models comprises a specific progenitor domain of the central nervous system ventricular zone, in which the process of motor neuron production is very well defined: the pMN domain of the spinal cord.

Dr. David J. Anderson's lab has been interested for several years in understanding the molecular control of the neuron-to-glia switch in this domain. Through these studies, Dr. Anderson's lab has developed the tools to carry out systematic gene expression profiling (GEP) experiments on purified oligodendrocyte progenitors, using Affymetrix microarrays. Preliminary data have been generated and candidate genes have been identified. CyclinD1, a positive cell cycle regulator, has been identified as specifically expressed in a subset of precursors, dynamically regulated during the neuronal to glia transition. This led us to hypothesize that CyclinD1 may regulate neurogenesis.

The experimental paradigm initially used is the modulation of its level of expression so as to monitor effect on the neurogenesis. CyclinD1 misexpression specifically increases the level of neurogenesis in the pMN domain, whereas RNAi-mediated CyclinD1 inhibition impairs neurogenesis. As a conclusion, we demonstrate that CyclinD1, independently of any effects on cell cycle, modulates the generation of MN. We believe this constitutes an unexpected result of potential importance for the field.

We are further testing the function of CyclinD1 using mouse-to-chick transplantations, which have been routinely done in Dr. Anderson's lab. We will also try to decipher the fundamental regulatory mechanisms by which CyclinD1 could trigger motoneuron fate specification. These studies will contribute to understanding the development of neuronal progenitor cell capacities, and their genetic control.

32. The role of neurosecretory cells in the modulation of *Drosophila* behavior

Timothy Tayler, Anne Hergarden

Animals exhibit countless complex and stereotyped behaviors such as aggression, courtship and the fight or flight response. These behaviors are likely generated and modulated by neural circuits. Other than a few simple reflex circuits, relatively little is known about how these circuits generate appropriate behaviors. *Drosophila* exhibits complex behaviors, but are anatomically less complicated and genetically more tractable than many vertebrate model systems. In addition,

flies have a highly developed set of molecular tools that can used to manipulate and analyze specific cell populations. Previous studies have demonstrated that *Drosophila* can be used to successfully identify neural correlates underlying complex behaviors such as courtship, olfactory aversion and learning and memory [1] [2] [3].

The goal of this project is to elucidate the connectivity, function, and modulation of circuits that underlie *Drosophila* behavior. To address this, we are attempting to manipulate neuropeptidergic neurons in the context of behavior. Neuropeptides are an important class of signaling molecules that are involved in a various aspects of animal physiology and behavior [4]. To gain genetic control over neuropeptide-producing neurons, we have identified the putative regulatory regions of 21 neuropeptide genes and have generated transgenic animals that will express the GAL4 protein under these promoters. The Gal4/UAS system allows for the expression of any of a large variety of transgenes in a spatial and temporally controlled manner. We are currently in the process of validating the expression patterns of these Gal4 lines while simultaneously using fluorescent reporters to characterize these neuropeptidergic neurons anatomically. In order to understand the role of these neuropeptidergic neurons in behavior, we will use genetic tools that will allow us to ablate, silence, or activate these neuropeptidergic neurons in the context of several behavioral paradigms.

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33. Neural circuitry of nociception in the skin and viscera

Sophia Vrontou, David J. Anderson

The brain detects noxious stimulation of the skin, viscera and other internal structures via different subsets of primary nociceptive sensory neurons. These subsets possibly engage distinct circuitry all the way up to the brain, explaining the ability to distinguish the bodily sites at which noxious stimuli are detected. Our objective is to get new molecular markers for such subsets and especially for viscera nociceptors that project to the gut and other internal organs and that form the basis of interoception, the brain's perception of the body's internal states. The ultimate goal is to use them, in conjunction with already identified markers for nociceptors innervating other targets, to trace their sensory maps, so as to illuminate whether these circuits are distinct labelled lines. This analysis might help deciphering the logic of brain decoding in the understudied visceral pain. In order to do that, we will dissect the circuitry, by gene targeting, of MrgB4/B5 members of the GPCR family of Mrgs, which is believed to be involved in nociception. Since the MrgD member is specifically expressed in cutaneous afferents, it is implied that the other Mrgs might be characteristic for nociceptor subsets innervating different organs, including viscera. Furthermore, since the purinergic receptor, P2X3, is expressed by both cutaneous and viscera nociceptive sensory neurons and MrgD neurons appear to innervate exclusively cutaneous targets, it follows that P2X3+ and MrgD- neurons are likely to contain visceral afferents. Thereby by creating appropriate targeted mutations in P2X3 and exploiting the already made in MrgD locus, we will be able to trace and compare the circuitry of the P2X3+ and MrgD- sensory neurons with that of P2X3+ and MrgD+. Additionally, in order to isolate new visceral nociceptor markers, we will conduct a screen that will be based on the separation by FACS and the comparison of the gene expression profile of cells from cutaneous nociceptors (marked by MrgD) and nociceptor afferents enriched by other markers for visceral targets (like c-RET, known to be expressed in P2X3 neurons).

34. Specific olfactory circuits in *Drosophila* direct an innate avoidance behavior

Greg S. Suh, David J. Anderson

We have developed a novel behavioral paradigm for an innate avoidance response in *Drosophila*. This paradigm involves avoidance of a substance (called *Drosophila* Stress Odorant or dSO) emitted by flies subjected to mechanical stress or electrical shock. Most responder flies, when given a choice in a T-maze between a fresh tube and a conditioned tube in which emitter flies were previously stressed, avoid the latter tube.

Gas Chromatography and Mass Spectrometry (GC/MS) and Respirometer analyses indicated that CO_2 is a component of dSO. Consistent with this, flies exhibited an avoidance response to CO_2 in a dosage-dependent manner. Surgical removal of the 3rd antennal segment, which houses olfactory receptor neurons (ORNs), rendered the flies defective in responding to dSO and CO_2, indicating that ORNs are likely required for avoidance to dSO and CO_2. In collaboration with Richard Axel's laboratory, we next identified a single pair of glomeruli in the antenna lobe that are innervated by Gr21a+ ORNs, that are specifically activated by CO_2 and are necessary for CO_2 avoidance (Suh *et al*., 2004).

We have mapped groups of neurons essential for the avoidance response to dSO, by carrying out an unbiased neuronal inactivation screen using the Gal4 x UAS-*Shibire*^{ts} system. We have screened ~200 Gal4 enhancer trap lines and have identified 14 lines defective only at the non-permissive temperature. In one of the 14 lines, c761, the avoidance response to CO_2, was also defective at the non-permissive temperature. In this line, Gal4 is expressed in Gr21a+ ORNs, consistent with the lack of CO_2-responsiveness. Moreover, the observation that c761 is expressed in additional populations of ORNs projecting to other glomeruli besides V, taken together with the lack of dSO responsiveness in c761; UAS-*Shi*^{ts}

flies, suggests that these ORNs may include those responding to another non-CO_2 component of dSO (NCO).

These lines of evidence indicated that dSO is composed of an unidentified substance(s) in addition to CO_2. To identify the groups of ORNs activated by NCO, we expressed GCaMP using pan-neuronal Elav-Gal4 and c761 drivers, and monitored the patterns of glomerular activation to traumatized fly air. We observed that another single pair of glomeruli, known as Dm1, was activated by traumatized fly air, but not by undisturbed fly air. The Dm1 glomeruli are innervated by Or42b+ ORNs. Using a combination of Or42b-Gal4, Gr21a-Gal4, and UAS-Shits transgenes, we showed that conditional inactivation of Or42b+ *and* Gr21a+ ORNs made flies defective in responding to dSO, whereas silencing either Or42+ or Gr21a+ ORNs was not sufficient. These results suggested that the two ORN populations act in combination and together are essential for dSO avoidance.

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35. Neuronal control of locomotor activity in the fruitfly

*Allan M. Wong, David J. Anderson, Michael H. Dickinson**

The fruitfly when presented with visual or olfactory stimuli responds with a change in behavior. For instance, when exposed to an expanding pattern of visual stimuli, the fly will move away from the focus of expansion; when exposed to low concentrations of carbon dioxide the fly will move away from the gas. In order to accomplish these behaviors, the fly must detect environmental changes, decide what to do, and coordinate its muscles to accomplish the behavior. We wish to understand how these steps are encoded in the nervous system of the fly. We reason a good entry point is to understand how locomotor information is encoded by the descending interneurons. These neurons connect the brain with the thoracic ganglia (the equivalent of the spinal cord in vertebrates), all visual and olfactory mediated behaviors must be transmitted through these neurons.

We have performed preliminary experiments to identify these neurons using photoactivable GFP. We expressed PA-GFP in all neurons in the fly, focally activated the neck connective, and then examined the brain to reveal about 90 pairs of neurons whose cell bodies are in the brain, fitting the descending interneuron definition.

We plan to record the activity of the descending interneurons while the fly is freely walking. In order to accomplish this, we built a walking arena modeled after Erich Buchner's design, where a tethered fly is walking on top of a floating ball. This allows for a preparation that simulates walking behavior while the fly is stationary, permitting functional imaging and electrophysiological recording with a two-photon microscope. We are also integrating the ball with the virtual flight arena hardware developed by Michael Riser to present visual stimuli to the walking fly.

We are also identifying enhancer trap lines that genetically label descending interneurons. We screened about 500 lines in the Heberlein collection and found five potentially useful lines. More detailed characterization of these lines is on going. Meanwhile, we have collaborations with other labs to screen through their enhancer trap collections. These lines will be useful for functional imaging studies by labeling a limited subset of neurons. This allows for a better signal to noise reading with functional imaging and targeted recordings with electrophysiology.

**Professor of Bioengineering, Caltech*

36. Investigation of behavior and neural circuitry underlying mechanosensation in *Drosophila melanogaster*

Suzuko Yorozu David Anderson

Mechanosensation is intricately linked to an organism's survival. For example, the ability to detect wind allows many flying insects to navigate, while other insects detect disturbances of air particles to help them locate predators and prey. Thus, a coordinated locomotor output in response to mechanical stimuli is critical for survival of organisms in nature. However, the link between mechanosensation and locomotor control at the neural circuit level is currently unknown. To address this question, we are trying to understand the neural circuitry underlying air current detection using a novel behavioral assay known as Air Suppression of Locomotion (ASL). In the ASL assay, wild-type adult flies immediately slow their locomotion and eventually stop moving when they are exposed to gentle air current. They remain immobile for as long as they are exposed to air flow stimulation, while they immediately re-engage in locomotor activity when the air flow stops, as if air flow stimulus acts as a neural switch for fly's locomotor activity. Interestingly, when third antennal segment is surgically removed bilaterally, they continue to engage in locomotor activity in the presence of air flow stimulation, suggesting that detection of air current is probably mediated through sensory organs housed in antennae. We propose to take advantage of the simplicity, robustness, high reproducibility, and unique locomotor phenotype of ASL to give insight into the link between mechanosensation and locomotor control at neural circuit level.

The goal of my project is to understand where and how air currents act on the nervous system to suppress locomotor activity in fly. To this end, we will combine behavioral genetics and functional analysis to address the following specific aims: 1) determine which sensory organ mediates ASL; and 2) identify the second- and higher-order neurons that mediate the processing of air current sensation and/or suppression of locomotor activity.

37. *In vivo* reversible silencing of striatal neurons by an ivermectin gated Cl channel

Walter Lerchner, Cheng Xiao, Laurent van Trigt, Eric M. Slimko, Henry A. Lester, David J. Anderson*

To develop tools for molecularly dissecting defined neuronal circuits in the brain, we have tested a two component ion-channel-based silencing system: expression of an ivermectin (IVM)-gated glutamate-sensitive Cl channel from *C. elegans*. The heteropentameric channel requires both *alpha* and *beta* subunits, allowing for combinatorial strategies to increase the specificity of expression. Both subunits have been codon optimized, rendered insensitive to glutamate and tagged with CFP (*alpha*) and YFP (*beta*) [1]. To test the system in the CNS *in vivo*, we injected mice unilaterally in the striatum with a suspension of two recombinant viruses (AAV2) expressing the *alpha* and *beta* subunits. IVM-induced silencing was confirmed in a simple rotation assay. Ten days after virus injection the mice were injected intraperitoneally with IVM and tested at various time points for amphetamine-induced rotation. If dopaminergic transmission via the striatum is unilaterally disrupted in amphetamine-injected animals, ipsilateral rotation occurs. Such rotation was observed in animals co-injected with virus encoding the *alpha* and *beta* subunit, but not with virus encoding the *alpha* or *beta* subunit alone. With injections of 10 mg/kg IVM, the rotation appears after 4 h, peaks at 12 h and remains amphetamine inducible for up to 3 d. We repeated IVM administration and withdrawal at least twice in the same animals. While repeated IVM administration restores the full rotation phenotype, in several mice some amphetamine-inducible rotation remains after IVM withdrawal. Further experiments will determine whether this effect is caused by behavioral sensitization, or by a percentage of neurons that cannot be reactivated after prolonged silencing. Preliminary striatal slice patch recordings show that virus-expressing neurons respond to a <1 min IVM pulse with hyperpolarization and reduced firing, as expected from of Cl channel activation. The conductance then reverses, which suggests that the several days required for reversal in behavioral experiments may arise from ivermectin pharmacokinetics and could be accelerated.

**Professor, Division of Biology, Caltech*

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38. Modeling "emotional" behavior in *Drosophila*

Tim Lebestky

Emotional behaviors in humans convey a positive or negative response to a stimulus, and this response is manifest in discrete, highly conspicuous ways, such as stereotyped facial expressions and physiological arousal. Although *Drosophila* do not present the richness of human emotions in their behavior, they may share fundamental molecular similarities that could allow us to dissect the way that neural circuits function to provide graded responses in intensity, as measured quantitatively. To this end, we are developing automated, high-resolution behavioral assays that will allow a reproducible characterization of behavioral responses to aversive stimuli for high-throughput genetic screens. One such assay follows the startle effects on locomotion and escape behaviors in response to a series of air puffs, delivered at regular intervals. We observe a reproducible escalation of locomotor activity and jump-response behaviors as a function of time and puff number. We have performed genetic screens to isolate and characterize insertional mutants and potential neural circuits that mediate startle behavior.

Similar to mammals, *Drosophila* utilizes biogenic amines as neurotransmitters for normal neuronal function and behavior. A serotonin transporter, dSERT, with significant functional homology to the mammalian SERT family has been cloned and physiologically characterized *in vitro* [1], however, there is no genetic analysis of this, or any other aminergic transporter *in vivo*. Given the importance of this molecular family and the successful advancement of *in vivo* RNAi techniques in *Drosophila*, we are currently developing techniques to look at gain- and loss-of-function conditions in a spatially and temporally regulated manner in adult flies. To this end, we have obtained RNAi lines, an insertional mutation in the dSERT locus, and multiple deficiencies that uncover the region. It is our hope that by tightly controlling the gene dosage and induction level of the RNAi, we may observe quantifiable phenotypic differences in behaviors that may give insights into how molecular thresholds influence the escalation or decline of distinct internal states in adult animals.

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39. Olig target genes in the oligodendrocyte-astrocyte fate decision

Christian Hochstim, Qiao Zhou, David J. Anderson

This study aims to contribute to the understanding of the molecular mechanisms of cell fate determination in central nervous system development. The more specific focus will be on the glial subtype choice between oligodendrocyte and astrocyte fates.

Olig2 is a bHLH transcription factor that has been shown to promote oligodendrocyte fate and repress astrocyte fate (Zhou and Anderson, *Cell*, 2002). In Olig2 -/- mice prospective oligodendrocytes marked by a GFP knockin are converted to astrocytes. In order to uncover downstream target genes of Olig2 involved in glial cell fate regulation we conducted gene expression profiling on Affymetrix microarrays using FACS isolated GFP+ cells from Olig2 +/- and Olig2 -/-. The paired homeodomain transcription factor Pax6 was identified as a gene

upregulated in the Olig2 mutant. Expression data confirms that the conversion of Olig2 -/- cells to astrocytes is correlated with the upregulation of Pax6 and that Pax6 is normally expressed in a subpopulations of astrocyte precursors and differentiated astrocytes but not in oligodendrocytes. Preliminary functional studies indicate that Pax6 is required for early astrocyte gene expression within a subset of precursors as well as for normal development of astrocytes in the ventral white matter of the spinal cord.

40. The amygdala central nucleus in innate and conditioned fear

Wulf Haubensak, David J. Anderson

Fear is probably the most conserved emotion, underlying defensive behaviors across species, and, in turn, it is a basic, medically important, human emotion that can be addressed in experimentally tractable animal model systems. Numerous studies have pointed to a central role of medial temporal lobe structures, particularly the amygdala, in various forms of fear. Typically, these structure-function relationships have been obtained by mapping patterns of neuronal activity accompanying fear associated behaviors, and functionally validating these correlations by surgical or neurotoxic lesions. However, these methods are not suitable to investigate single neuronal circuits with cellular resolution. This is especially important when it comes to assign function to the amygdala subregions, and of the different neuronal populations therein. Among these, neurons expressing the stress promoting neuropeptide CRH in the central amygdala (CeA) are likely to have a central role in fear processing.

Here, we explore a genetic strategy in mice to analyze function and circuitry of these neurons with higher precision.

CRH is, as many other genes, expressed in more than one brain region. We, therefore, use it in combination with another region-specific gene, *PKC delta,* which is only coexpressed with CRH in the CeA, and thereby restrict the expression of inducible genetically-encoded neuronal silencers specifically to CeA neurons. To this end, we generated *CRH* and *PKC delta* transgenic mice expressing each one of the two subunits of a chloride channel for silencing (Slimko *et al., J. Neurosci.* **22**:7373), such that in intercrosses of these mice, the functional channel will be reconstituted only in the target cells. This will allow us to silence these cells in a temporally-defined manner in behavioral paradigms for innate (measuring ultrasound-induced freezing in foot-shock sensitized mice [1] and conditioned (measuring freezing in tone/foot-shock conditioned mice) fear.

We will then use stereotactic injections of Cre-dependent viruses for neuronal tracing into the CeA of a *PKC delta*-Cre transgenic mouse line to identify projections to and from these neurons.

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Summary: Our group uses *Drosophila* as a model system in which to identify and characterize genes involved in behavior, aging, and neurodegeneration. The high degree of homology between the fly and human genomes forms the basis of a strategy for understanding the corresponding human genes.

To study the genetics of aging, we use a single-gene approach to screen for mutants with enhanced longevity, and analyze the functions of the genes involved. For instance, the mutant, *methuselah*, extends the average lifespan of *Drosophila* by some 30% and also provides increased resistance to different stresses. The methuselah protein is related to G protein-coupled receptors of the secretin receptor family, and has a unique N-terminal ectodomain. In collaboration with Professor Richard Roberts' group, a library of peptides was generated, from which a subset was selected that show very high binding affinity to Mth protein. Overexpression of some of these peptides in transgenic flies extends lifespan.

Exposure of flies to 100% oxygen causes rapid death, and we have found that an early-induced event is local disruption of muscle mitochondrial structure in the form of internal "swirls." These also accumulate in normal aging. We have identified and are characterizing mutants that are unusually sensitive to oxygen and display large numbers of swirls, as well as degeneration of specific cell types in the nervous system. Analysis of such mutants may provide clues to primary mechanisms of oxidative damage. Mutations in specific components of the respiratory chain are of special interest in relation to human mitochondrial disorders.

Dietary restriction extends lifespan in various organisms, and we are investigating the mechanism of that phenomenon in *Drosophila*, as well as the role of bacterial flora. We have developed biomarkers to monitor the progress of aging during lifetime, and have shown that lifespan can be extended by simple feeding of a drug which alters the balance of induction and repression of different sets of genes.

Among the behavioral paradigms currently under investigation are an alarm response in which flies subjected to stress emit an odor that induces avoidance by other flies, a genetic analysis of appetite and obesity, and the effects of a sex peptide in *Drosophila* sperm that revamps the behavior of mated females.

41. Biochemical characterization of a *Drosophila* mitochondrial complex II mutant

David W. Walker, Petr Hájek, Julien Muffat, Dan Knoepfle, Giuseppe Attardi[*]*, Seymour Benzer*

In a screen to identify genes that protect the mitochondrion from oxidative stress, we discovered a fly mutant with a defect in subunit B of succinate dehydrogenase (SDH; mitochondrial respiratory complex II). Mitochondrial complex II, the only respiratory chain complex that is completely encoded by nuclear genes, consists of four subunits; SDHA, SDHB, SDHC and SDHD. Inactivation of complex II manifests a range of disorders, including familial pheochromocytoma, and familial head-and-neck paraganglioma. Understanding of these diseases has been limited by a dearth of animal models carrying mutations in each of the complex II subunits.

Under hyperoxia (100% O_2), the mean survival of *sdhB* mutant flies, is reduced by 91%. Under normoxic conditions, mutant flies displayed a 66% decrease in mean survival time and a 17% decrease in maximum survival time. To determine the effect of the *sdhB* mutation on respiratory chain activity, the rate of oxygen consumption was measured, using a Clark-type oxygen electrode. The steps in respiration were compared in mitochondria isolated from wild-type and from *sdhB* mutant flies, using complex-specific substrates and inhibitors. Complex I-specific (rotenone-sensitive) respiration rate was normal in the *sdhB* mutant as was the Complex IV-specific (ascorbate/TMPD-dependent/KCN-sensitive) respiration rate. However, there was a 56% decrease in Complex II-specific (succinate-dependent/antimycin A-sensitive) respiration rate.

The effect of the mutation on production of reactive oxygen species (ROS) was assayed by mitochondrial hydrogen peroxide production. Mitochondria from *sdhB* mutant animals had a 32% increase in hydrogen peroxide production, as compared to wild-type controls. Our data indicate that SDHB is critical in preventing electron leakage from complex II, so that mutant animals suffer from increased oxidative stress and, as a result, are highly sensitive to oxygen and die rapidly. To determine the consequences of increased levels of SDHB, we generated transgenic flies that overexpress the normal gene. Mean lifespan was increased by 50% in flies that overexpress *sdhB*.

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42. *sdhB* gene activity is protective in a *Drosophila* model of ischemia/reperfusion injury

David W. Walker, Seymour Benzer

When tissues and organs are deprived of blood flow, the ischemic effects result from inadequate nutrients and oxygen. When blood flow is restored (reperfusion), a second series of injurious events is triggered. Ischemia and reperfusion can cause serious brain damage as a result of stroke or cardiac arrest. Several studies have demonstrated that the reactions initiated at reperfusion involve the formation of cytotoxic oxidants derived from molecular oxygen.

Biochemical characterization of a fly mutant with a defect in subunit B of succinate dehydrogenase (SDH; mitochondrial respiratory complex II) reveals that *sdhB* mutants suffer from increased mitochondrial oxidative stress. To determine whether normal *sdhB* activity is important in protecting against reperfusion injury, we tested the ability of *sdhB* mutants to withstand periods of oxygen deprivation, by subjecting them to 100% N_2, followed by recovery under normoxia. Groups of 20-25 adult flies were exposed to anoxic conditions (100% N_2), in which the flies quickly lost coordination and became motionless. After 45 minutes, they were allowed to recover in room air. We compared the ability of wild-type and mutant flies to recover from this anoxic stress. Normal flies began to waken after 8 minutes; by 24 minutes, all were awake. *sdhB* mutants displayed a delay in recovery from anoxia, beginning to wake after 13 minutes, but at 24 minutes, when all control flies had awakened, less than 50% of the mutant flies had. By 1 hour, all had recovered, but the *sdhB* mutant flies were still much less active than controls. We measured the flies' climbing performance before and after the hypoxia/reperfusion paradigm. Young mutant flies, at 2 days of age, which in normoxia had climbed quite well, afterward displayed a severe climbing defect, not seen in normal flies. These data indicate that *sdhB* is indeed protective against reperfusion injury and suggests that mutant animals can serve as a general model for studying mechanisms of reperfusion injury.

43. Neurophysiological analysis of aging in *Drosophila*

Shlomo Ben Tabou de Leon, David W. Walker, Seymour Benzer

Most studies on aging in *Drosophila* are based on survival curves, without regard to the physiological changes that underlie mortality. To that end, we are studying age-related changes in electrophysiological function of specific organs, including the flight muscles and the photoreceptors, in normal flies and mutants. The role of mitochondrial dysfunction is studied by using the mutant *sdhB*, which is defective in subunit B of complex II of the mitochondrial electron transport chain. This mutant is short lived and shows mitochondrial dysfunction with age and elevated ROS production.

We find that in normal flies both muscle fibers and photoreceptors show strong deterioration in electrophysiological function with age. In muscle fibers, the resting potential decreases and the action potential amplitude becomes smaller. Photoreceptors show a decrease in response to light, as manifested by decreases in both the receptor potential and the "on" and "off" responses in the electroretinogram. The eyes also develop spontaneous activity (in the dark). Electron microscopy reveals associated retinal degeneration. Under oxidative stress (100% O_2), muscle fibers of normal and *sdhB* mutant flies showed similar deterioration, but the photoreceptors of the mutant flies showed less deterioration than normal flies. The differential response of these two tissues to oxidative stress suggests that increased ROS level may not be sufficient to account for all the deterioration of function with age. Investigating the molecular basis of tissue-specific deterioration may provide clues about causes of death, and specifically the role of the mitochondria.

44. Modeling the role of Apolipoprotein D in normal and pathological aging in *Drosophila*

Julien A. Muffat, David W. Walker, Seymour Benzer

We have been investigating the roles of Apolipoprotein D and its fly homologs *Glial Lazarillo* (*GLaz*) and *Neural Lazarillo* (*Nlaz*) under aging, conditions of stress, and in pathological situations. In Alzheimer's disease the human protein is strongly upregulated and recruited to senile plaques. More recently, it has been shown also to be upregulated in the substantia nigra of Parkinson's disease patients, at the site of degenerating dopaminergic neurons. While human ApoD (hApoD) is upregulated in many situations of neuronal damage, research has yet to determine whether it is part of a stress response meant to control and repair the damage, or a marker of and actor in cell death. Our goal has been to use the powerful genetic tools available in *Drosophila* to modulate the expression of the homologs of ApoD in flies.

We recently reported that overexpression of *GLaz* extends lifespan of flies by up to 30% under normal conditions, while also increasing resistance to multiple extrinsic stresses. Most importantly, healthspan is also improved, as assessed by behavioral assays. For example, normal flies exposed to hyperoxia for 4 days display a decline in their ability to climb, while flies overexpressing *GLaz* are protected against this decay, and continue to climb normally. *Glaz* overexpression also appears to protect flies from a behavioral decline induced by a hypoxia/reoxygenation treatment thought to model stroke. This result is particularly important considering that ApoD is upregulated in a mouse model of stroke.

GLaz loss-of-function flies are sensitive to oxidative stress and short-lived. We are currently testing UAS-RNAi lines for both *GLaz* and *NLaz*. These will allow us to study time and tissue-specific down-regulation of the two genes. We can then find out which tissues are

necessary for the normal functions of *GLaz* and *NLaz*. An important experiment will be to try to rescue neurodegeneration in mutants with the human protein.

We generated several constructs placing the sequences of *GLaz*, *NLaz* and *hApoD* under UAS control in a modified pUASt plasmid. The plasmids were designed to fuse a C-terminal tag (Ha or GFP) to the different proteins. We selected, for each construct, one with the gene inserted on the 2nd chromosome, and one on the 3rd chromosome. Anti-HA staining on protein preparations from transformants shows clear immunoreactive bands, of the expected molecular weight. We find that flies overexpressing *NLaz* or *hApoD* are 30 to 40% more resistant to the oxidative stress caused by paraquat, as well as living longer under normal conditions. These results are evidence of functional homology between *GLaz/NLaz* and *hApoD* and suggest that their functions are beneficial.

45. Doubling of fruitfly lifespan by overexpression of a mitochondrial gene

William W. Ja, Gil B. Carvalho, Seymour Benzer

Hypomorphic mutations in either the G protein-coupled receptor (GPCR) gene *methuselah* (*mth*) or its ligand *stunted* result in robust extensions of *Drosophila* lifespan. Surprisingly, we have found that ubiquitous overexpression of Stunted during development, but not during adulthood, leads to an unprecedented two-fold extension of adult longevity. Stunted encodes a critical component of the mitochondrial ATP synthase, implying a connection between metabolism, the ATP-producing machinery, and GPCR signaling in the lifespan-extending effect. The long-lived animals overexpressing Stunted show reduced size, body mass and fertility, suggesting a tradeoff between metabolism and longevity. We are currently dissecting the role of Stunted as a regulator of energy investment, and determining the connection between Methuselah signaling and mitochondrial metabolism.

46. Downstream mediators of dietary restriction

Brian M. Zid, Pankaj Kapahi, Seymour Benzer*

Dietary restriction (DR), the reduction of calories without malnutrition, has been known to extend lifespan in mice since the 1930's and has since been shown to work in a variety of organisms. DR is also the most potent, broadly acting intervention for preventing the age-related disease cancer. DR causes a shift in an organism's physiology; in *Drosophila*, this is characterized by fat accumulation, increased stress resistance, and reduced fertility. We have found that the eIF4E binding protein, 4EBP, is dramatically upregulated as the concentration of yeast extract in the fly food is reduced. 4EBP is necessary for the lifespan extension seen under DR as male and female 4EBP mull flies show a diminished response to the reduction in YE concentration. Using the Gal4-UAS system, we overexpressed d4EBP using two separate weak, ubiquitous drivers and found that, in females, this upregulation extended lifespan on high YE but not under DR.

eIF4E is part of the cap binding complex that is necessary for the translation of the majority of mRNAs. mRNAs with long 5'UTRs that have a high amount of secondary structure are especially sensitive to the activity of eIF4E. Many mRNAs with high amounts of secondary structure are important in the control of cell growth and proliferation. The polysome profile of flies under DR was investigated, and translation state array analysis (TSAA) performed to assay the translational state of individual mRNAs. DR significantly changed the translational levels of 107 genes, 81 up and 26 down. To investigate whether there is a relation between 5'UTR secondary structure and translation regulation under DR, we calculated the folding free energy, ΔG, for the 5'UTR for each gene that changed significantly. It was found that the average 5'UTR of the upregulated genes was 92 bp long and had a ΔG of -13.9 kcal/mol, while the downregulated genes had average 5'UTR lengths of 324 bp and ΔG's of -71.1 kcal/mol. There was a highly significant translational downregulation of transcripts that had high secondary structure in their 5'UTR, consistent with the hypothesis that eIF4E activity is downregulated upon DR. Current research is focused on understanding the functional classification of the genes which translationally change under DR.

**Buck Institute, Novato, CA*

47. *creosote* mutants in *Drosophila* as a model to identify genetic suppressors of the toxic effects of over-nutrition

Jonathan Liong, William Ja, Pankaj Kapahi, Seymour Benzer*

Dietary restriction extends lifespan in various organisms. That implies that *ad libitum* food intake has a toxic effect. By feeding flies on excessively rich food, we can screen for genes that counteract this toxic effect. We designate mutants that are hypersensitive to mutation as *creosote* mutants. These can, in principle, provide models with which to screen for drugs and genes that act as suppressors of the deleterious effects of over-nutrition. Since the basic cellular processes and pathways are strongly conserved among organisms, and there is a high degree of genetic homology between *Drosophila* and humans, information obtained from *creosote* mutants may help to uncover the role of nutrition in the etiology of age-related human diseases. To identify *creosote* lines we compared adult lifespan on high yeast compared to low yeast foods (20% versus 5% brewer's yeast), using lines overexpressing RNAi of various genes. We discovered that down-regulation of a gene involved in electron transport, oxido-reductase, and monooxygenase activity causes sensitivity to high yeast food. We will verify whether down-regulation of the gene is responsible for the *creosote* phenotype, and will continue to screen a collection of RNAi lines for additional gene targets.

**Buck Institute, Novato, CA*

48. Obese mutants of *Drosophila*

Bader F. Al Anzi, Seymour Benzer

In a screen for obese mutants, we found *fatso*, which is recessive for both starvation resistance and obesity. Both phenotypes map to a 300 Kbp genomic interval, and we are currently doing complementation tests between *fatso* and other mutant genes in that genomic interval. Via various assays, we have examined the underlying basis of the phenotype. *fatso* has a 25% increase in food intake and also shows reduced locomotor activity. However, these are not associated with reduced metabolic rate, as indicated by CO_2 generation. In fact, there is a 10-15% increase in CO_2 emission with age. Fat also accumulates in the thoracic muscle. In the abdomen there are abnormally enlarged fat droplets with central nuclei.

In a second mutant, *Butterball*, both starvation resistance and obesity are dominant. *Butterball* shows a 50-60% increase in CO_2 emission, as compared to wild-type controls, which might indicate a metabolic abnormality. *Butterball* also exhibits abnormal fat cell morphology but, unlike *fatso*, does not show storage of fat in thoracic muscle with age. Surprisingly, unlike *fatso*, *Butterball* flies have 25-30% increase in lifespan as compared to wild-type controls.

Using a two-dye feeding assay to isolate mutants with abnormal feeding patterns, we found that a P-element insertion in the neuropeptide hormone *leucokinin* causes abdominal bloating, associated with increased food intake. Excision lines produced by mobilization of this P-element revert this phenotype, confirming that the bloating defect is indeed caused by the P-element insertion in the *leucokinin* gene.

Leucokinin is a myotropic neuropeptide found in most invertebrate species. It was initially isolated as a neurohormone that increases fluid secretion in the malpighian tubules, and also stimulates hindgut motility. The biological activity of Leucokinin hinges upon the presence of a C-terminal pentapeptide, a feature that it shares with the vertebrate tachykinin peptides, a family of peptides including molecules such as substance P (SP), known to be involved in gut motility, which is a likely vertebrate evolutionary ortholog of Leucokinin.

The known role of Leucokinin in stimulating fluid secretion of the malpighian tubules prompted us to examine whether *leucokinin* mutants exhibit any defect in maintaining osmotic balance. We addressed this issue by growing the mutant flies in media posing high osmotic stress. Unlike wild-type flies, *leucokinin* mutant adults begin to die within 24 hours after being transferred to media with a high concentration of NaCl, KCl or D-sorbitol. We are currently examining whether this phenotype in fact maps to the *leucokinin* gene.

49. Appetite mutants of *Drosophila melanogaster*

Paul Nagami, Bader F. Al Anzi, Seymour Benzer

We used a two-color assay to screen for mutants that overeat. In the two-color assay, flies are starved for 24 hours, then transferred for 20 minutes to red-dyed food, then transferred for 15 minutes to blue-dyed food. Flies that are not satiated in 20 minutes of feeding consume both red and blue food, and so have purple bellies and are potential overeaters.

We screened the *Drosophila* X chromosome for EMS-induced mutants with altered feeding behavior using the assay. In addition, we have screened some 60 lines with P-element-induced deletions in genes chosen as potential appetite regulatory genes. Candidate mutant lines obtained by these screens are being further characterized.

50. Odor sensitivity and habituation in a *Drosophila dNOS* mutant

Vickie Pon, Bader F. Al Anzi, Seymour Benzer

Nitric oxide (NO) is a gaseous signaling molecule that mediates the production of cyclic guanosine monophosphate (cGMP), a second messenger involved in many biological processes. NO is largely produced by the enzymatic activity of nitric oxide synthase (NOS), an essential enzyme in most animals. Among its many roles, NO is involved in odor habituation, a process that protects from sensory overload. We examined a *Drosophila* mutant with a P-element insertion in the gene that encodes NOS (called *dNOS*) to determine whether the mutant has any olfactory defects. Using diaphorase staining, we found that these *dNOS* mutants have a decrease in NO production in its antennae and maxillary palps, which are thought to be the main olfactory organs of the adult fly. By performing behavioral experiments using a T-maze, we next examined if this reduction in NO production is correlated with olfactory defects. We found that *dNOS* mutants are more sensitive to a variety of odors at lower concentrations than control flies. Odor habituation tests indicated that *dNOS* mutants do not habituate to most odors to the same degree as do control flies, suggesting that the increased sensitivity to odors in *dNOS* mutants may be due to the defect in habituation.

51. A search for "forgetless" mutants

Joseph Donovan, Bader F. Al Anzi, Seymour Benzer

While great strides have been made towards understanding memory, forgetting is still a poorly understood process. While some aspects of forgetting must involve passive processes, such as decay of neural activity over time, there is the possibility that an active forgetting mechanism exists. The goal of this project is to develop a new, more efficient paradigm for *Drosophila* olfactory memory conditioning assays, and to use the paradigm to search for mutants that fail to forget. The new paradigm differs from the conventional *Drosophila* olfactory paradigm in that flies are conditioned to reverse their behavior, i.e., to avoid a normally attractive odor.

This facilitates detection of mutants with long-lasting memory. Screening is in progress using the new paradigm, and there are several promising mutant lines, but more testing is required to determine whether they are truly forgetless mutants.

52. The effect of mating and seminal peptides on female food preference

Gil B. Carvalho, William W. Ja, Elizabeth Mak, Seymour Benzer

In numerous insect species, mating elicits a dramatic remodeling of behavior. In *Drosophila*, this post-mating response (PMR) is mediated by small peptides transferred in the male ejaculate. We have recently shown that copulation also induces a marked increase in female appetite, and that this change is controlled by a single male seminal factor, the Sex Peptide (SP). We have developed a procedure, the CAFE (capillary feeding) assay that allows one to directly monitor real-time ingestion and feeding preference. When presented with a choice between sugar and yeast, virgins ingest more of the sugary food but mated females show an enhanced preference for the latter. We are investigating the role of SP in regulating this paradigm.

53. Genetic analysis of action of the *Drosophila* sex peptide

George Luo, Gil Carvalho, Seymour Benzer

Drosophila sex peptide (SP) is synthesized in the adult male accessory gland and transferred via the seminal fluid to the female during copulation. SP thus induces profound changes in female reproductive behavior and physiology, including repressed sexual receptivity, increased egg-laying, reduced lifespan, increased feeding, and an alteration in food choices. To reveal the pathways underlying these post-mating responses, a key step is to identify the SP receptor(s). We are using a genetic screening assay that makes use of the dramatic reduction in female sexual receptivity after receiving SP. While normal females will not remate for many hours, females with a effect in the SP pathway should accept remating. We sequentially exposed females of various mutant lines to two groups of males, each carrying a distinctive genetic marker to identify the resultant progeny. With normal females, only ≈5% accepts a secondary mating. In contrast, some mutant lines show a much higher degree of acceptance. These data suggested that this screening assay is capable of identifying SP pathway-defective mutants.

54. Olfactory circuits in *Drosophila* avoidance of stress-associated odors

Greg S. Suh, Allan Wong[1], Anne Hergarden[1], Richard Axel[2], Seymour Benzer, David J. Anderson[3]

Recognition of many odorants in insects requires decoding of the combinatorial patterns of glomerular activation, mediated by the mushroom body (MB), which receives inputs from the most of projection neurons (PNs), called the inner antennocerebral tract. Other tracts bypass the MB and make direct connections to the inner brain. As CO_2 can elicit avoidance response by activating a single population of ORNs rather than a complex combinatorial set, it would be interesting to know whether decoding of the simple CO_2 olfactory information requires the MB function. The fact that the MB is not required for CO_2 avoidance, however, suggests that the CO_2 olfactory information is unlikely processed in the MB. It has been shown that locomotor activity triggered by acute exposure of CO_2 to flies does not habituate, while the activity elicited by other odors does habituate, which requires the MB function. Accordingly, repeated exposure of CO_2 to flies does not habituate the avoidance response in a T-maze assay. Furthermore, it has been difficult to train flies to conditionally associate CO_2 (conditioned stimuli) with electric shock (unconditioned stimuli). These results raise an interesting possibility that CO_2-responsive PNs bypass the MB and directly innervate the inner brain. To visualize the connectivity pattern of PNs innervated V (V-PN), we utilized a Gal4 enhancer trap line expressed in PNs including those innervated the V glomeruli. Using the FLP-out technique of Basler and Struhl, we showed that a V-PN bypasses the MB and directly innervates the laternal horn. The Axel lab subsequently showed that the V-PN labeled by the Gal4 line is activated by CO_2.

The lines of evidence indicate that dSO contains an unidentified substance(s) in addition to CO_2, "the mystery odor." To identify the groups of ORNs activated by the mystery odor, we expressed GCaMP using pan-neuronal Elav-Gal4 and c761 drivers, and monitored the patterns of glomerular activation to air from traumatized flies. We found that another single pair of glomeruli, known as Dm1, was activated by such air, but not by air from undisturbed flies. The Dm1 glomeruli are innervated by Or42b+ORNs. Using the batteries of Or42b-Gal4, Gr21a-Gal4, and UAS-Shits transgenes, we showed that conditional inactivation of Or42b+ *and* Gr21a+ ORNs made flies defective in responding to dSO.

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Summary: Research in the laboratory focuses on three areas: (1) Biophysics of computation in nerve cells; (2) understanding visual selective attention and visual consciousness at the neuronal, behavioral and computational levels; and, (3) based on this understanding, develop biological-motivated vision algorithm and apply them to the automatic recognition of overhead imagery, robotic navigation and other machine vision tasks. For more details and all publications, see http://www.klab.caltech.edu

Research carried out as part of a "Biophysics of Computation" focuses on how the electrophysiology, synaptic architecture, and dendritic morphology of groups of individual neurons subserve information processing. To what extent do neuronal noise sources (thermal and channel noise, noise due to synaptic background firing, and so on) limit signal detection and signal reconstruction at the level of individual neurons? What are the biophysical mechanisms underlying neuronal computations? Can we infer something about the shape and property of a neuron by listening to its extracellular observed action potential? How does the constantly fluctuating extracellular membrane potential influence the intracellular membrane potential? Can the extracellular membrane potential carry information of relevance to neural computation? Analytical work, backed up by detailed computer simulations of nerve cells based on electrophysiological data from our experimental collaborators at the Hebrew University in Jerusalem (Idan Segev and Yosef Yarom) and Henry Lester at Caltech, generates experimentally verifiable predictions. With Guyri Buzsaki at Rutgers, we continue to investigate the relationship between extra- and intracellular spike waveforms and with Tomaso Poggio at MIT we are identifying ways in which single neurons can implement specific neuronal operations, such as computing the maximum over a cell' synaptic input (MAX).

Understanding the action of selective, visual attention (both saliency-driven, bottom-up, as well as task-dependent, top-down forms) requires a firm grasp of how visual object recognition in natural scenes can be solved at the computational level, and how the resulting algorithms can be mapped onto the known architecture of the visual cortex and associated subcortical areas. We use analytical methods, coupled with computer simulations of the appropriate circuitry in the primate visual system, experimental psychophysics, eye trackers and functional brain imaging to investigate human object recognition in the near-absence of focal attention, in visual search and in natural scene perception (some of this is done in collaboration with Laurent Itti at USC). Together with Tomaso Poggio at MIT, we investigate neurobiologically plausible models of both the ventral and dorsal visual streams. What are the limits of human object recognition? To what extent can probabilistic Bayesian recognition algorithms perform near-human level visual object classification (the object is a dog) and identification (it's my dog Bella). This is ongoing work with Pietro Perona at Caltech. The resulting algorithms are being applied to problems in overhead imagery analysis, robotic navigation and scene interpretation for real-time machine vision.

We are complementing these studies using noninvasive fMRI imaging (at Caltech's 3.4 T scanner) under the identical stimulus protocols to investigate such questions as object recognition with and without spatial selective attention, as well as orientation and face-specific adaptation.

Our laboratory collaborates with the neurosurgeon and neuroscientist Itzhak Fried at UCLA, recording from 64 electrodes in the medial temporal lobe of awake patients with pharmacologically intractable epilepsy who are implanted with depth electrodes to localize the focus of seizure onset. This unique setting allows us to observe invariant recognition, imagery and representation of familiar objects and famous individuals in conscious humans by listening in on the spiking activity of many individual neurons - complemented by local field analysis. This work requires sophisticated data processing skills and the careful design of the appropriate behavioral-physiological paradigms that will work in a clinical context.

Finally, we continue to study the neuronal correlates of consciousness, developing a neurobiological framework to understand how subjective feelings (in particular, conscious visual perception) can arise in the mammalian forebrain. This two decade-long research

program has found its way into a book for a general scientific audience, *The Quest for Consciousness: A Neurobiological Approach*, by C. Koch and published in 2004.

55. On the origins of the extracellular action potential waveform: A modeling study

Carl Gold[1], Darrell A. Henze[2], Christof Koch[1], György Buzsáki[3]

Although extracellular unit recording is typically used for the detection of spike occurrences, it also has the theoretical ability to report about what are typically considered intracellular features of the action potential. We address this theoretical ability by developing a model system that captures features of experimentally recorded simultaneous intracellular and extracellular recordings of CA1 pyramidal neurons. We use the Line Source Approximation method (Holt and Koch, 1999) to model the extracellular action potential (EAP) voltage resulting from the spiking activity of individual neurons.

We compare the simultaneous intracellular and extracellular recordings of CA1 pyramidal neurons recorded *in vivo* (Henze *et al.*, 2000) with model predictions for the same cells reconstructed and simulated with compartmental models.

The model accurately reproduces both the waveform and the amplitude of the EAP's, although it was difficult to achieve simultaneous good matches on both the intracellular and extracellular waveforms. This suggests that accounting for the EAP waveform provides a considerable constraint on the overall model. The developed model explains how and why the waveform varies with electrode position relative to the recorded cell. Interestingly, each cell's dendritic morphology had very little impact on the EAP waveform. The model also demonstrates that the varied composition of ionic currents in different cells is reflected in the features of the EAP.

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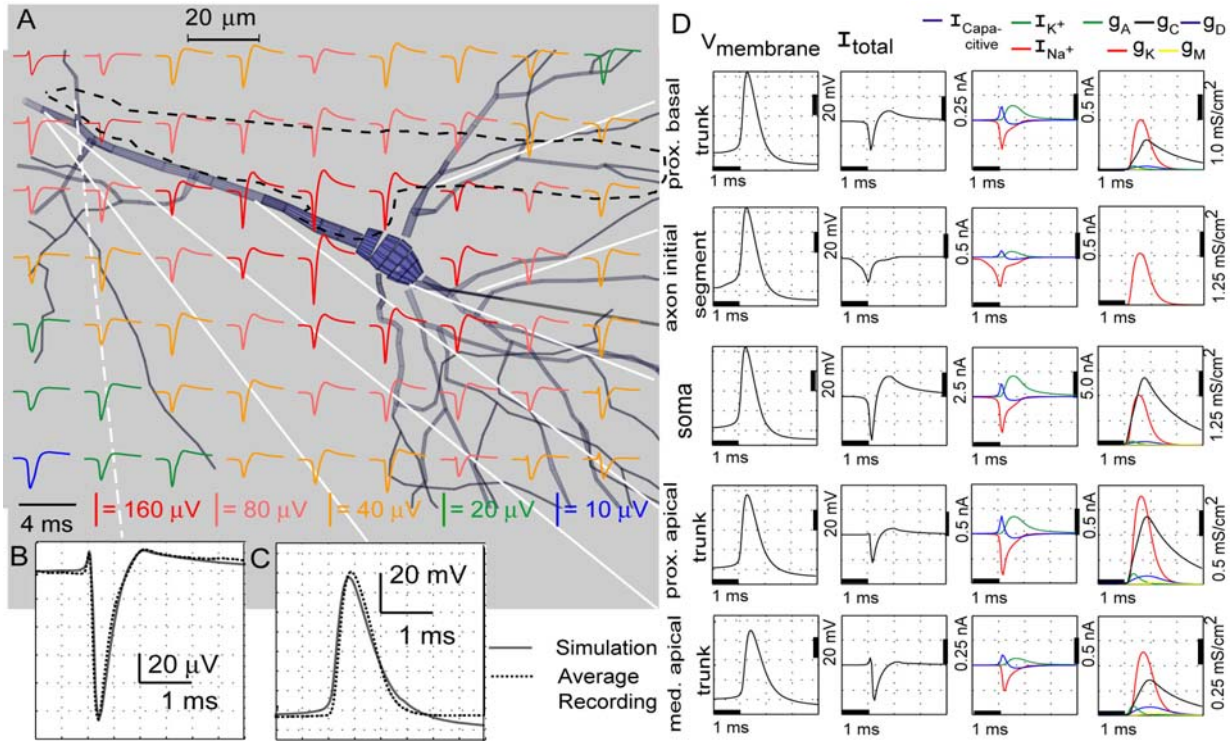

A: EAP's in the transverse section containing the soma and the tip of the electrode track (dotted line). **B**: Enlargement of the EAP at the estimated electrode position, and comparison to the recording (strongest channel of the tetrode). **C**: Comparison of the average intracellular recording with the simulated spike in the proximal apical trunk. **D**: Details of the simulation in the indicated compartments. The shape of the EAP waveform is given by the shape of the net membrane current across the membrane at the soma and proximal dendrites (second column).

56. Spike propagation in dendrites with stochastic ion channels

Kamran Diba[1], Christof Koch, Idan Segev[2]

We investigate the effects of the stochastic nature of ion channels on the faithfulness, precision and reproducibility of electrical signal transmission in weakly active, dendritic membrane. The properties of forward and backpropagating action potentials (BPAPs) in the dendritic tree of pyramidal cells are the subject of intense empirical work and theoretical speculation. We numerically simulate the effects of stochastic ion channels on the forward and backward propagation of dendritic spikes in Monte-Carlo simulations on a reconstructed layer 5 pyramidal neuron. We report that in most instances there is little variation in timing or amplitude for a single BPAP, while variable backpropagation can occur for trains of action potentials. Additionally, we find that the generation and forward propagation of dendritic Ca^{2+} spikes are susceptible to channel variability. This indicates limitations on computations that depend on the precise timing of Ca^{2+} spikes.

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57. Model of extracellular electrode detection range and sampling bias

Carl Gold[1], C. Girardin[2], John Anderson[2], Rodney Douglas[2], Christof Koch

We simulate the biophysics of intra- and extracellular current flow to calculate the distance at which an extracellular electrode could record neurons from different layers of cat visual cortex. Detailed 3-D reconstructions of neurons from identified layers of cat visual cortex (N=70; Binzegger *et. al*., 2004) coupled to a detailed electrophysiological membrane model, were simulated using compartmental models within NEURON (Hines and Carnevale, 1997). We use the Line Source Approximation (Holt and Koch, 1999) to model the extracellular action potential voltage resulting from the spiking activity of individual neurons (Gold *et. al*., 2006). The peak extracellular voltage resulting from an action potential was calculated in the vicinity of each model cell. A threshold determined by the background noise was used to calculate a region in which an extracellular electrode would be able to detect the action potential. For each layer of cortex we calculated an average detection range for a typical neuron located in that layer. We found that the size of the detectable region around a cell depended strongly on the size and morphology of the cells. Based on the average detection volume, we calculated the number of cells that would be within range of a fixed electrode for each layer using previous measurements of the density of cells in each layer (Beaulieu and Colonnier, 1983). The number of cells within detection range varied from only 2 in layer 4 to around 20 in layer 5. (Layers 2/3 and 6A were more similar to layer 4; see Table 1.) This calculation is roughly in agreement with previous empirical measurements, which found an average of 4.4 units detectable by tetrodes *in vivo* (Gray *et. al*., 1995.) We also calculated the sampling bias of each layer, assuming that the fraction of cells in each layer firing at any given time was uniform. This calculation suggests that the probability of sampling from any layer, given an electrode randomly placed somewhere in the grey matter, is influenced by both the size of the different layers and the detection range around the cells of that layer.

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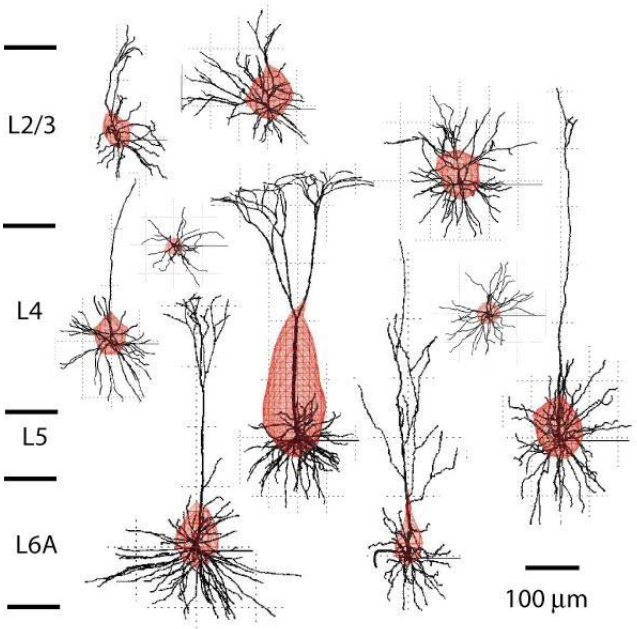

Illustration of detection regions around cells from different layers of cat visual cortex.

58. Using extracellular action potential recordings to constrain parameters of detailed compartmental models

Carl Gold[*], Christof Koch

We compare two different methods for constraining the active current conductance density parameters of detailed compartmental models. The first method is using a measurement of the intracellular action potential (IAP) at one or more locations on the membrane. The second method is to calculate the Extracellular Action Potential (EAP) from the membrane currents, using the Line Source Approximation (LSA). We find that using the IAP as the parameter has the drawback that significantly different sets of parameters can have virtually identical IAP's. In contrast, the EAP's for the same simulations vary considerably as a result of the parameter differences. The reason for the phenomena is that the IAP results from both membrane currents on a compartment and also axial current owing from neighboring compartments. The EAP results from the local membrane currents alone. This gives the IAP additional degrees of freedom in comparison to the IAP. Based on these results we conclude that use of the EAP to constrain compartmental model parameters may be superior to using measurements of the IAP.

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59. Local field potentials and spikes in the human MTL are selective to image category

Alexander Kraskov, Rodrigo Quian Quiroga[1], Leila Reddy[2], Itzhak Fried[3], Christof Koch

Local field potentials (LFPs) reflect the averaged dendro-somatic activity of synaptic signals of large neuronal populations. In this study we investigated the selectivity of local field potentials and single neuron activity to semantic categories of visual stimuli in the medial temporal lobe of nine neurosurgical patients implanted with intracranial depth electrodes for clinical reasons. Strong selectivity to the category of presented images was found for the amplitude of LFPs in 8% of implanted microelectrodes and for the firing rates of single and multi units in 14% of microelectrodes. There was little overlap between the LFP- and spike-selective microelectrodes. Separate analysis of the power and phase of LFPs revealed that the mean phase was category-selective around the frequency range and that the power of the LFPs was category-selective for high frequencies around the γ rhythm. Of the 36 microelectrodes with amplitude-selective LFPs, 30 were found in the hippocampus. Finally, it was possible to read-out information about the category of stimuli presented to the patients with both spikes and LFPs. Combining spiking and LFP activity enhanced the decoding accuracy in comparison with the accuracy obtained with each signal alone, especially for short time intervals.

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60. A single neuron correlate of change detection and change blindness in the human medial temporal lobe

Leila Reddy[1], Rodrigo Quian Quiroga[2], Patrick Wilken[3], Christof Koch, Itzhak Fried[4]

Observers are often unaware of changes in their visual environment when attention is not focused at the location of the change. Because of its rather intriguing nature, this phenomenon, known as change blindness, has been extensively studied with psychophysics as well as with fMRI. However, whether change blindness can be tracked in the activity of single cells is not clear. To explore the neural correlates of change detection and change blindness, we recorded from single neurons in the human medial temporal lobe (MTL) during a change detection paradigm. The preferred pictures of the visually responsive units elicited significantly higher firing rates on those trials when subjects correctly identified a change (change detection) compared to when they missed it (change blindness). On correct trials, the firing activity of individual units allowed us to predict the occurrence of a change, on a trial-by-trial basis, with 67% accuracy. This prediction was at chance for incorrect trials. Furthermore, subjects' behavioral choices on each trial could be predicted above chance (58%) based on these individual responses. The firing rates of visual selective MTL cells thus constitute a neural correlate of change detection.

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61. Invariant visual representation by single neurons in the human brain

Rodrigo Quian Quiroga[1], Leila Reddy[2], Gabriel Kreiman[2], Christof Koch, Itzhak Fried[3]

We can easily recognize a person or object even if viewed from different angles or under strikingly different conditions. How such a robust high-level representation is achieved by neurons in the brain is still unclear and has led to different hypothesis. We analyzed neuronal responses in the human medial temporal lobe to presentations of images of individuals and objects. Using a new spike-sorting algorithm, we extracted spiking activity from intracranial recordings in the temporal lobe of patients with pharmacologically intractable epilepsy. These patients were implanted with depth electrodes, in combination with multiple micro-wires, in order to localize the seizure focus area for potential surgical resection. From a total of 64 micro-wires, usually located in the amygdala, entorhinal cortex, hippocampus and parahippocampal gyrus, we isolated between 40 and 90 simultaneously recorded units per experiment. For each patient, we first analyzed single- and multi-unit responses to presentations of about 100 different pictures, comprising famous and non-famous people, animals, landmarks, and objects. Then, we selected those images for which at least one unit showed a significant response and, in subsequent

sessions, we presented different views of those individuals, animals, or objects. From a total of 998 units recorded in eight patients, 51 units were activated by different presentations of an individual, landmark or object, with few or no responses to images of other individuals or objects. Some of these neurons also responded selectively to the letter string with the name of the individual. Noteworthy, these responses were in many cases limited to short bursts occurring between 300-600 msec. These results point towards a sparse, invariant and explicit representation of individuals or objects by neurons in the human temporal lobe.

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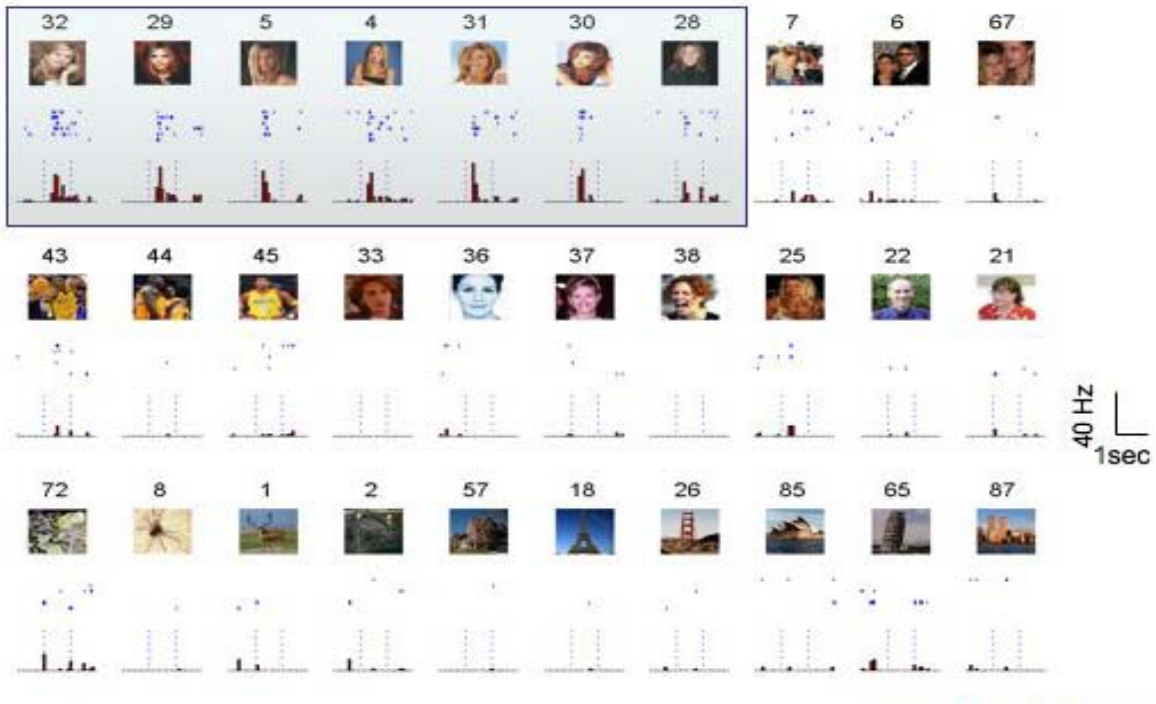

Selective and invariant responses in human neurons: Spiking responses and histograms of a single unit in the left posterior hippocampus of a human subject activated exclusively by different views of the actress Jennifer Aniston. Only the responses to 30 of the 87 images shown to the patient are given. There were no statistically significant responses to the other 57 pictures.

62. Computational modeling of sparse representation

Stephen Waydo[1], Alexander Kraskov, Rodrigo Quian Quiroga[2], Itzhak Fried[3], Christof Koch

Recent experiments characterized individual neurons in the human medial temporal lobe (MTL) with remarkably selective, invariant and explicit responses to images of famous individuals or landmark buildings. We use probabilistic analysis to show that these data are consistent with a sparse code in which neurons respond in a selective manner to a small fraction of stimuli. We further develop a computational model based on a current state-of-the-art machine vision system (Hmax) that is able to generate category selective behavior after an unsupervised learning process.

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63. Linearity of color- and luminance-contrast in human overt attention

Sonja Engmann[1,2], Thomas Sieren[2], Selim Onat[2], Peter König[2], Wolfgang Einhäuser

When inspecting a complex natural scene, human observers sequentially allocate their attention to subsets of the stimulus for detailed processing. Since these shifts in attention are usually associated with shifts in gaze, eye-position provides an overt correlate of the focus of attention. While several factors, such as the observer's experience and the task, influence the direction of gaze under natural conditions, most models of human attention concentrate on the influence of bottom-up factors, i.e., features of the current stimulus. Most of these models use the concept of a so-called saliency map (Koch and Ullman, 1985). They compute center-surround differences independently in multiple feature channels, normalize the difference maps in each feature individually and add the resulting "conspicuity maps" linearly across features. While such models predict human fixation above chance, it

is unclear whether their two fundamental assumptions, linearity and independence of features, do indeed hold.

Here we investigate the contribution of two-specific features, color-contrast and luminance-contrast, to human overt attention in natural scenes. We measure eye-position of observers viewing stimuli where luminance-contrast and/or color-contrast are increased gradually towards one side of the image. We find that such gradients bias fixation locations towards regions of higher contrasts. If both features increase in the same or in opposing direction, the individual biases add linearly. In addition, both features act independently as revealed by perpendicular feature gradients.

These findings demonstrate that the effects of color- and luminance-contrast on human overt attention are consistent with the predictions and assumptions of bottom-up saliency map models.

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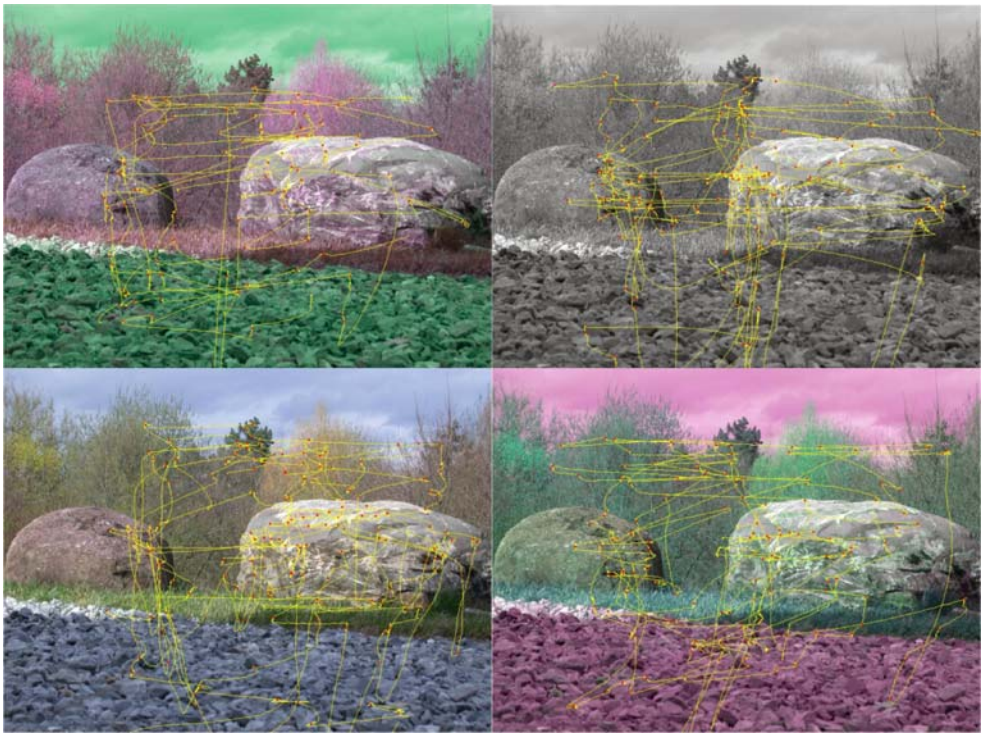

64. The duration of the attentional blink in natural scenes depends on stimulus category

Wolfgang Einhäuser, Christof Koch, Scott Makeig[*]

Humans comprehend the "gist" of even a complex natural scene within a small fraction of a second. If, however, observers are asked to detect targets in a sequence of rapidly presented items, recognition of a target succeeding another target by about a third of a second is severely impaired. Remarkably, this impairment is absent if the second target immediately follows the first. These two phenomena constitute the "attentional blink" (AB; Raymond, Shapiro, and Arnell, 1992). Since most experiments on the AB use well controlled but artificial stimuli, the question arises whether the same phenomenon occurs for complex, natural stimuli, and if so, whether its specifics depend on stimulus category. Here we presented rapid sequences of complex stimuli (photographs of objects, scenes and faces) and asked observers to detect and remember items of a specific category (either faces, watches, or both). We found a consistent AB for both target categories but the duration of the AB depended on the target category.

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65. Phase-noise and overt attention in natural scenes

Wolfgang Einhäuser, Ueli Rutishauser[1], Swantje Nadler[2], Peter König[2], Christof Koch

Under natural conditions, humans sequentially allocate their attention to subsets of the stimulus. Models of attention are typically based on difference maps in low-level features, but neglect higher-order statistics inherent in natural stimuli. To what extent does such higher-order structure affect the deployment of attention? Here we recorded eye-movements in human observers who viewed unmodified and modified images of natural scenes. Modifications included contrast modulations (resulting in changes to first and second-order statistics), as well as the addition of noise to the Fourier phase (resulting in changes to higher-order statistics). We find: First, subjects' interpretation of a stimulus as a "natural" depiction of an outdoor scene depends on higher-order statistics. Second, a linear relation between fixation probability and first-order feature contrast predicts the observed fixation distribution, if the feature is modulated independently of the underlying scene. However, for features originally present in the scene, this relation depends on the interplay between spatial frequency and higher-order statistics. This holds for various stimulus categories (man-made, faces, out-door scenes and fractals). Taking both findings together, our data imply that the effect of first-order features is related to scene interpretation. Consequently, a linear model explains the relation of attention to first-order features, if they occur in isolation. For natural scenes, however, higher order correlations have to be taken into account.

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66. Task-demands can reverse the effects of visual saliency

Wolfgang Einhäuser, Christof Koch

In natural vision, stimulus features and the demands of the task affect observers' attention and eye movements. The exact relationship between sensory-driven and task-dependent factors is controversial. On the one hand, "bottom–up" models that rely solely on sensory-driven features (e.g., "saliency-maps") successfully predict a significant fraction of free viewing fixations. Models of visual search, on the other hand, focus on task-related ("top-down") factors: they predict the selective up-modulation of target features or filters, or an optimized strategy according to general scene statistics. We demonstrate a robust effect of sensory-driven saliency under free viewing conditions that can, however, be completely and immediately overridden by a search task.

67. Modeling human errors in rapid scene recognition

T. Nathan Mundhenk[1], Wolfgang Einhäuser, Christof Koch, Pierre Baldi[2], Laurent Itti[1]

Humans demonstrate a peculiar ability to detect complex target items (such as an animal) in sequences of rapidly presented natural scenes. They thereby far outperform contemporary machine vision algorithms, yet little computational understanding exists of the neural mechanisms underlying such rapid visual processing. To characterize these mechanisms and to translate them into a computational model, we quantitatively analyze the conditions under which rapid target detection succeeds and fails in humans. Three observers viewed 1000 sequences of 20 natural scene images, presented at 20Hz. They performed a yes/no paradigm as to whether one image of an animal was embedded in the sequence, which was the case in half of the trials. Consistently with earlier studies, subjects performed well above chance. In addition, performance of different observers was not mutually independent: in more sequences than predicted by chance did all three observers detect the target ("hit": 207/500 trials) or miss it ("miss": 93/500 trials). This implies that the spatio-temporal statistics of a sequence allow prediction of target detection success. We quantify this using two computational vision algorithms: one computes instantaneous visual surprise, an information-theoretic measure of attentional saliency, while the other evaluates the distribution of image contrast. We find that consistently detected targets have significantly higher contrast than targets that are consistently missed. More importantly, images immediately preceding and following a target elicited significantly more visual surprise in "miss" than in "hit" trials. This suggests that items that parasitically capture attention impair target detection by effectively masking the target. Using these measures, we demonstrate that a neural network can predict task performance on a trial-by-trial basis. Being the first computational model predicting human performance in rapid visual detection tasks, our results pave the way for improving machine vision algorithms on the basis of understanding the mechanisms of human performance.

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68. Visual motion area MT+ responds to auditory motion in early blind subjects

Melissa Saenz, Ione Fine[1], Alex Huth[2], L.B. Lewis[*], Christof Koch

Much of the human brain cortex is devoted to visual processing, leading to the question of what happens to the visual cortex in people who are blind? Numerous studies have demonstrated cross-modal responses to auditory and tactile stimuli within human visual cortex as a result of early blindness. However, the mechanisms underlying this cross-modal plasticity are still not well understood. One critical question that has not yet been

addressed is how these cross-modal responses within reorganized visual cortex map onto "normal" cortical organization.

Here we demonstrate an example of cross-modal plasticity in blind humans in which the reorganized cortical visual area maintains its functional role. Using functional magnetic resonance imaging (fMRI), we found that visual area MT+, well established for its role in visual motion processing in sighted subjects, is selectively recruited for auditory motion processing in early blind subjects. We defined areas that responded selectively to moving auditory stimuli in seven sighted subjects, two formerly blind subject with partial sight recovery, and five early-blind subjects. We verified area MT+ location (human medial temporal area, thought to be homologous to macaque areas MT/V5 and MST) in the five sighted subjects and two formerly blind subjects using a standard visual MT+ localizer scan. Within both early blind subjects and formerly blind subjects we found significant responses to auditory motion within medial temporal areas that were defined as area MT+ using a conjunction of anatomical and functional criteria. Sighted subjects did not show responses to auditory motion within MT+.

These data suggest that area MT+ continues to fulfill its specialized computational role of motion processing, even in the absence of visual input. This is, to our knowledge, the first demonstration that cross-modal reorganization may map onto pre-existing functions of cortex, suggesting an organizational principle behind cross-modal plasticity whereby cortical regions, despite receiving information from a novel modality, remain "functionally constant."

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69. Combined effects of spatial and deature-based Attention

Melissa Saenz, Geoff Boynton[*]*, Christof Koch*

Spatial and feature-based attention are known to modulate cortical visual responses, but little is known about how these (or other) forms of attention interact when combined. Using fMRI in humans, we measured the individual and combined effects of spatial and feature-based attention in visual cortex. Subjects (n=5) viewed two circular apertures of moving random dots presented to the left and right of central fixation. The test side had one field of dots moving either upward or downward. The other side had two overlapping fields of dots moving upward and downward. Subjects performed a threshold-level speed discrimination task on one of the three dot fields at a time. We measured the modulation of the response to the *test* field under three attention conditions. In the spatial condition (S), attention alternated between the test field and the field with the same motion direction on the other side (block design). In the feature-based condition (F), attention alternated between the fields with the same and opposite motion directions on the other side. In the S+F condition, attention alternated between the test field and the field with the opposite direction on the other side. In areas V1, V2, V3, V3A, V4, and MT+, the response modulation caused by changing both the attended location and the attended feature (S+F) equaled the sum of that caused by changing the attended location (S) and the attended feature (F) alone. Thus, spatial and feature-based attention mutually reinforce their effects in the earliest stages of cortical visual processing.

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70. Attention and consciousness: Two distinct brain processes

Christof Koch, Naotsugu Tsuchiya[*]

The relationship between attention and consciousness is a close one. In many cases, we become conscious of what we attend to. This leads many scholars to conflate the two. We here summarize psychophysical evidence arguing that top-down attention and consciousness are distinct phenomena that need not occur together and that can be manipulated using distinct psychophysical paradigms. Subjects can become conscious of an isolated object, or the gist of the scene in the near absence of top-down attention. Conversely, subjects can attend to perceptually invisible objects. Most remarkable, top-down attention and consciousness can have opposing effects. Such dissociations became easier to understand when one considers the different functions of these two processes.

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71. Dissociating microgenesis of retinal and non-retinal adaptation

Naotsugu Tsuchiya[1]*, Lee A Gilroy*[2]*, Randolph Blake*[2]*, Christof Koch*

Negative afterimages have traditionally been ascribed to retinal adaptation, but recent studies suggest that adaptation mediating afterimage formation occurs at multiple levels of visual processing. Here, we use an interocular suppression technique, called continuous flash suppression (Tsuchiya and Koch, 2005), to dissociate microgenesis of retinal adaptation (which should be immune to suppression) from non-retinal adaptation (which is susceptible to suppression).

We achieve this dissociation by holding retinal adaptation constant at 5 sec while manipulating the duration of suppression. Remarkably, presentation of five, brief flashes during the last 500 msec of adaptation was sufficient to reduce the magnitude of non-retinal adaptation by half. To quantify the strength of suppression, we used a forced-choice probe detection task and found that as few as five successive, brief flashes interfered with probe detection as much as continuous flash suppression.

However, a single flash only exerted suppression within a narrow window of time between onset of the probe and onset of the suppression figure; moreover, the suppression from this single flash did not sum its effect when we paired flash suppression with conventional binocular rivalry suppression.

This modified flash suppression technique provides a useful method for dissecting adaptation arising within mechanisms at multiple visual processing levels.

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Reference

Tsuchiya, N. and Koch, C. (2005) *Nat. Neurosci.* **8**:1096-1101.

72. Depth of interocular suppression associated with continuous flash suppression, flash suppression, and binocular rivalry

Naotsugu Tsuchiya[1], Christof Koch, Lee A Gilroy[2], Randolph Blake[2]

When conflicting images are presented to the corresponding regions of the two eyes, only one image may be consciously perceived. In binocular rivalry, two images alternate in phenomenal visibility; even a salient image is eventually suppressed by an image of low saliency. Recently, (Tsuchiya and Koch, 2005) reported a technique called continuous flash suppression (CFS), extending the suppression duration more than 10-fold. Here, we investigated the depth of this prolonged form of interocular suppression, as well as conventional binocular rivalry and flash suppression using a probe detection task. Compared to monocular viewing condition, CFS elevated detection thresholds more than 20-fold, while binocular rivalry did so by 3-fold. In subsequent experiments, we dissected CFS into several components. By manipulating the number and timing of flashes with respect to the probe, we found that the stronger suppression in CFS is not due to summation between binocular rivalry and flash suppression, but is caused by the summation of the suppression due to multiple flashes. Our results support the view that CFS is not a stronger version of binocular rivalry, but is due to the accumulated suppressive effects of multiple flashes.

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[2]*Department of Psychology, Vanderbilt University*

Reference

Tsuchiya, N. and Koch, C. (2005) *Nat. Neurosci.* **8**:1096-1101.

73. Continuous flash suppression reduces negative afterimages

Naotsugu Tsuchiya[*], Christof Koch

Illusions that produce perceptual suppression despite constant retinal input are used to manipulate visual consciousness. Here we report on a powerful variant of existing techniques, continuous flash suppression. Distinct images flashed successively at ~10 Hz into one eye reliably suppress an image presented to the other eye. The duration of perceptual suppression is at least ten times greater than that produced by binocular rivalry. Using this tool we show that the strength of the negative afterimage of an adaptor was reduced by half when it was perceptually suppressed by input from the other eye. The more likely the adaptor was completely suppressed, the larger the reduction of the afterimage intensity. Paradoxically, trial-to-trial visibility of the adaptor did not correlate with the degree of reduction. Our results imply that formation of afterimages involves neuronal structures that access input from both eyes, but that do not correspond directly to the neuronal correlates of perceptual awareness

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74. The continuous wagon wheel Illusion is associated with changes in EEG power at 13 Hz

Rufin VanRullen[1], Leila Reddy[2], Christof Koch

Temporal subsampling of the perceptual stream can cause illusory reversals of the perceived motion direction. This "Wagon Wheel Illusion," most apparent in movies or on television, can also be observed under continuous illumination, suggesting that our visual systems too might sample motion in a sequence of discrete epochs (Purves *et al*., *Proc. Nat. Acad. Sci. USA*, 1996). This phenomenon is bistable by nature, with the actual motion direction generally dominating perception (Kline *et al., Vis. Res*. 2004).

Psychophysics: Previously we reported that a motion energy model subsampling visual inputs at a rate between 10 and 20 Hz can quantitatively predict the relative durations of real and illusory percepts during continuous viewing of the illusion (VanRullen *et al.,* Society for Neuroscience, 2004). Here we use pairs of gratings drifting in opposite directions to investigate this effect. By way of these "counterphase" gratings of slightly different contrasts, one can directly manipulate the ambiguity of motion direction, and enhance the relative strength of the illusory percept. We find that motion direction judgments for these stimuli are selectively impaired around 10 Hz, as predicted by the temporally subsampled motion energy model. This impairment vanished when focal attention was directed away from the motion stimulus: we used a dual-task paradigm to draw spatial attention to a stream of rapidly presented randomly rotated letters at the center of the grating. Under these conditions, simultaneous motion direction judgments were in fact better at 10 Hz than when attention was directed to the motion itself. This is one of very few known instances were focal attention is found to impair performance. These results support the idea that, at least in some circumstances, the visual system represents motion in discrete epochs, and that this effect is mediated by focal attention.

EEG: We then tested 12 observers who watched a rotating wheel (on a 160 Hz monitor, fast enough to avoid contamination by framing artifacts) for thirty, two-minute long trials. 32-channel EEG activity was recorded concurrently. Subjects continuously reported the perceived direction of motion by pressing the arrows on a keyboard. Two stimulus temporal frequencies were used (7.5 and 10 Hz). Illusory motion occurred more than 30% of the time on average, consistent with previous reports.

We separated periods of perceived actual and perceived illusory motion, and compared the EEG power spectrum (from 2 to 70 Hz) in these two conditions. The only reliable difference was observed over centro-parietal electrodes at approximately 13 Hz: EEG power in this range was significantly higher (corrected for multiple comparisons across electrodes) during periods of perceived real motion than during illusory periods. This difference at 13 Hz, and the corresponding scalp topography, was observed for both stimulus temporal frequencies, and thus probably reflects internal processes rather than stimulus-related activity. 13 Hz EEG power decreased prior to the onset of illusory motion, and increased before transitions back to real motion. Using this relation it was possible to predict above chance, on a trial-by-trial basis, the direction of the upcoming perceptual transition, with prediction accuracy for some subjects above 70%. We discuss potential mechanisms for the generation of the illusion, and the relation to our previous model.

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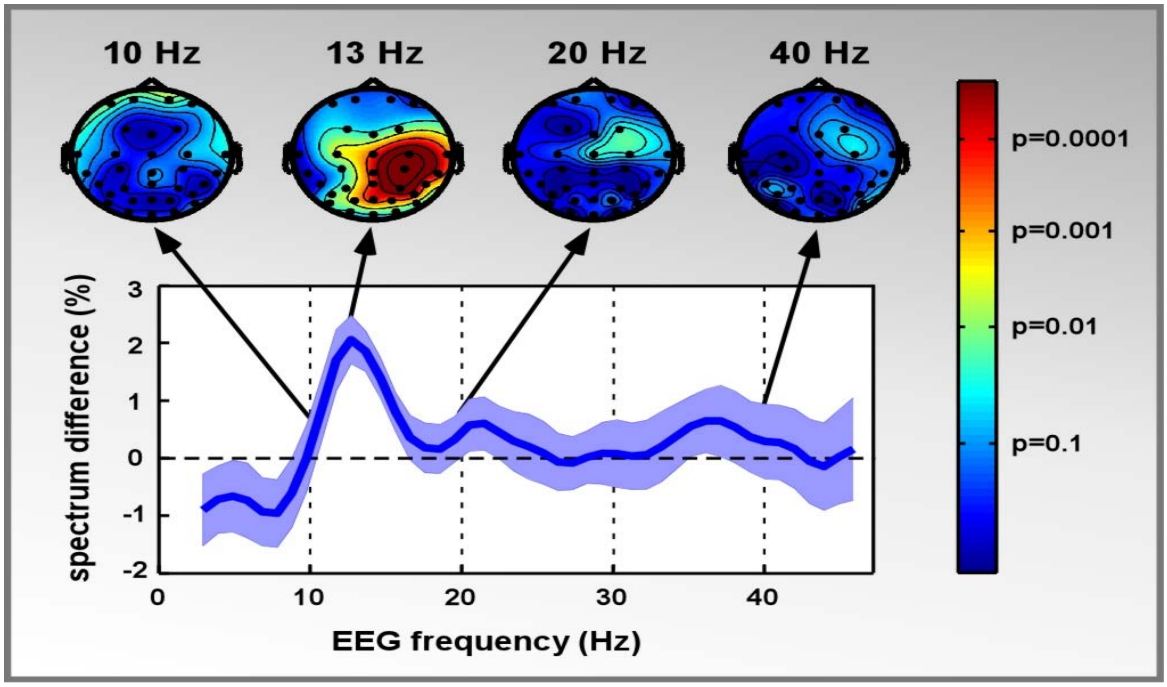


Electrophysiological correlates of the continuous Wagon Wheel Illusion (c-WWI). We compared the power spectrum of the EEG during periods when subjects experienced real or illusory motion on a wheel rotating at a fixed frequency (the results were comparable when this frequency was 10Hz or when it was 7.5Hz). Even though the physical stimulus was identical in both situations, one component of the power spectrum (and one only) differed significantly between the two conditions. This component was centred around 13Hz, exactly as predicted by our model of the c-WWI based on periodic attentional sampling of motion information. The localization of this effect over right parietal regions is compatible with the known involvement of these regions in focusing attention to temporal events.

75. Explicit and implicit processes in human aversive conditioning

Ronald McKell Carter, Christof Koch

The ability to adapt to a changing environment is central to an organism's success. The process of associating two stimuli (as in associative conditioning) requires very little in the way of neural machinery. In fact, organisms with only a few hundred neurons show conditioning that is specific to an associated cue. This type of learning is commonly referred to as implicit learning. The learning can be performed in the absence of the subject's ability to describe it. One example of learning that is thought to be implicit is *delay conditioning.* Delay conditioning consists of a single cue (a tone, for example) that starts before, and then overlaps with, an outcome (like a pain stimulus).

In addition to associating sensory cues, humans routinely link abstract concepts with an outcome. This more complex learning is often described as explicit since subjects are able to describe the link between the stimulus and outcome. An example of conditioning that requires this type of knowledge is *trace conditioning.* Trace conditioning includes a separation of a few seconds between the cue and outcome. Explicit learning is often proposed to involve a separate system, but the degree of separation between implicit associations and explicit learning is still debated.

We describe aversive conditioning experiments in human subjects used to study the degree of interaction that takes place between explicit and implicit systems. We do this in three ways. First, if a higher order task (in this case a working memory task) is performed during conditioning, it reduces not only explicit learning but also implicit learning. Second, we describe the area of the brain involved in explicit learning during conditioning and confirm that it is active during both trace and delay conditioning. Third, using functional magnetic resonance imaging (fMRI), we describe hemodynamic activity changes in perceptual areas of the brain that occur during delay conditioning and persist after the learned association has faded.

From these studies, we conclude that there is a strong interaction between explicit and implicit learning systems, with one often directly changing the function of the other.

76. Contingency awareness in conditioning involves the middle frontal gyrus

Ronald McKell Carter, Christof Koch

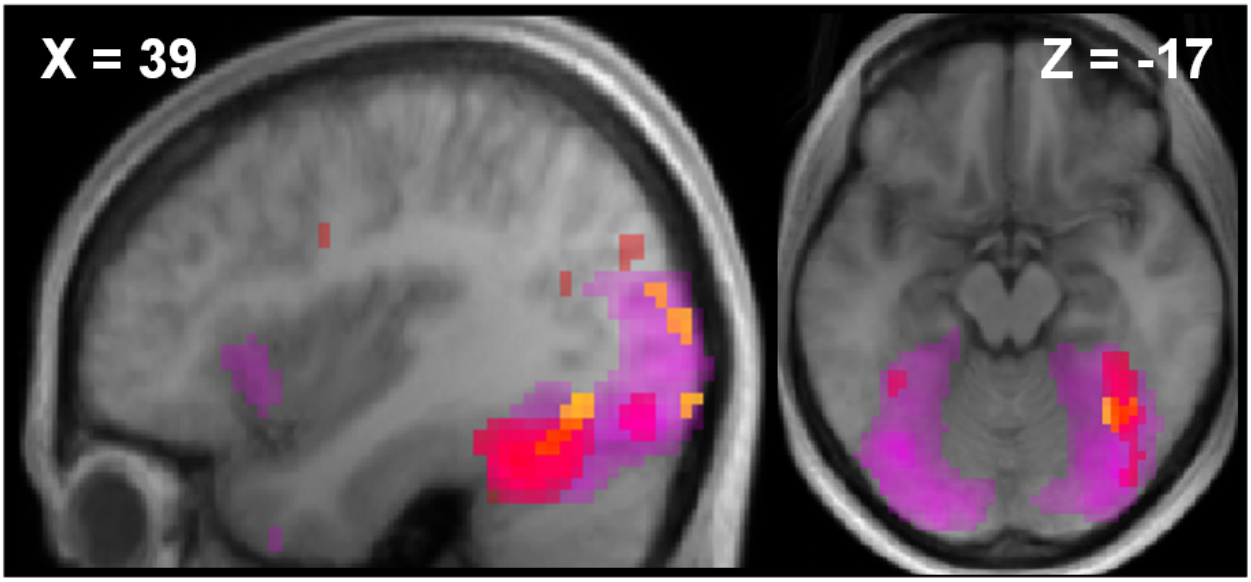
In contrast to the wealth of data describing the neural mechanisms underlying classical conditioning, we know remarkably little about the mechanisms involved in acquisition of explicit contingency awareness. Subjects variably acquire contingency awareness in classical conditioning paradigms, in which they are able to describe the temporal relationship between a conditioned cue and its outcome. Previous studies have implicated the hippocampus and prefrontal cortex in the acquisition of explicit knowledge, although their specific roles remain unclear. We used functional magnetic resonance imaging to track the trial-by-trial acquisition of explicit knowledge in a concurrent trace and delay-conditioning paradigm. We show that activity in bilateral middle frontal gyrus and parahippocampal gyrus correlates with the accuracy of explicit contingency awareness on each trial. In contrast, amygdala activation correlates with conditioned responses indexed by skin conductance responses (SCRs). These results demonstrate that brain regions known to be involved in other aspects of learning and memory also play a specific role, reflecting on each trial the acquisition and representation of contingency awareness.

77. Aversive conditioning results in persistent changes in the fusiform face area

Ronald McKell Carter, Christof Koch

In classical conditioning, an initially neutral stimulus acquires significance by being repeatedly paired with a stimulus that has affective value – such as an aversive shock. Studies on the neural basis of conditioning have focused on the role of structures such as the amygdala in mediating learning of these associations. A fundamental question that has received less attention is whether neural representations of the conditioned stimulus itself are modified through conditioning, and if so where in the stimulus processing pathway such changes take place. Here, we address whether neural representations of a specific class of visual stimuli: faces, can be modulated as a function of conditioning, and if so, whether such changes persist in extinction.

We scanned xx human subjects using fMRI while undergoing a differential conditioning paradigm in which presentation of one of two faces was repeatedly paired with aversive shocks. Analysis of BOLD activity in the fusiform face area revealed increasing activity in this area to the conditioned face when compared to the neutral face. This is contrary to the phenomenon of repetition suppression where repeated presentations of a visual stimulus normally show decreased activation over time. Moreover, activity in this area is resistant to extinction, and persists well beyond diminution of conditioned skin conductance responses. We conclude that aversive conditioning results in an increase in representation of a conditioned visual stimulus that persists beyond the extinction of physiological fear responses.



Summary of changes in face representation over conditioning and extinction. BOLD activity in the fusiform face area (displayed above in red) increases during acquisition of conditioning (displayed above in yellow). This activity is persistent throughout extinction of the conditioned association (displayed above in magenta). Activity is displayed using a lower threshold than previous figures (P<0.01 uncorrected) to indicate the extent of overlap.

78. Face identification in the near-absence of focal attention

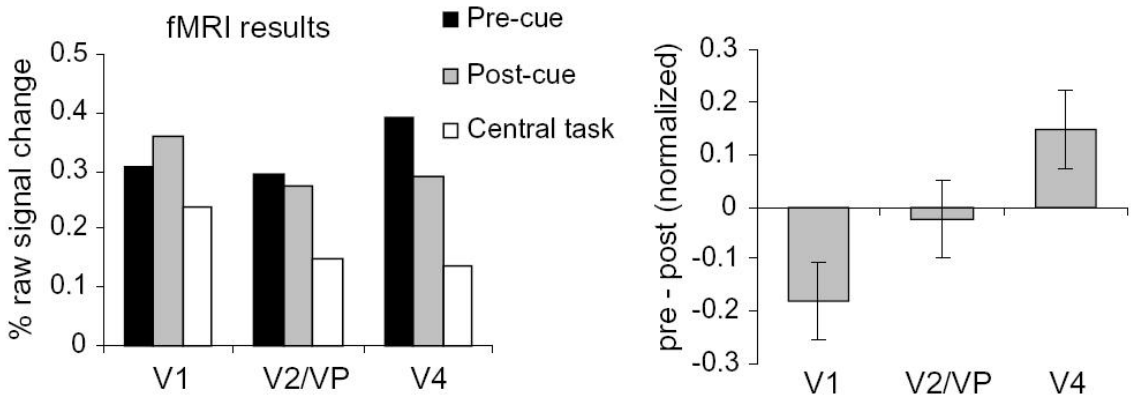
Leila Reddy, Lavanya Reddy, Christof Koch

In contrast to artificial geometric shapes, natural scenes and face-gender can be processed even when spatial attention is not fully available. In this study, we investigate whether a finer discrimination, at the level of the individual, is possible in the near-absence of focal attention. Using the dual-task paradigm, subjects performed face-identification on faces of celebrities and relatively unfamiliar individuals, along with a task that is known to engage spatial attention. We find that face-identification performance is only modestly impaired under dual-task conditions. These results suggest that the visual system is well able to make complex judgments of natural stimuli, even when attention is not fully available.

79. Attention enhances stimulus-driven feedforward activity in human visual cortex

Farshad Moradi, Christof Koch

Attention evokes fMRI response in striate and extrastriate areas even in the absence of retinal input. It remains unclear whether stimulus-dependent activation also increases with attention. We dissociated stimulus-dependent effects from changes in baseline response by directing attention either before, or after, a peripheral grating is displayed. Directing attention before stimulus onset should increase both baseline activity and the activity evoked by the grating. Post-stimulus attention should increase baseline activity but not the activity evoked by the grating. Two square-wave gratings were displayed in the upper quadrants at 10 deg. A brief central cue instructed subjects either to report the orientation of one of the two gratings, or to perform a central demanding task (control). The cue appeared 400 ms before or 250 ms after the onset of the stimuli. Seven observers participated. Directing attention to the peripheral gratings enhanced BOLD activity (measured in a 3.0T Siemens scanner) in V1-V4 compared to control conditions (Figure). Subjective discrimination and V4 BOLD activity were significantly enhanced under pre-stimulus cue conditions compared to post-stimulus cue. Contrariwise, V1 activity was significantly higher under post-stimulus attention than under pre-stimulus attention. Enhancement of V4 response to the attended grating (despite less V1 activity in the same condition) is in agreement with the hypothesis that attention facilitates feed-forward sensory processing by increasing neural gain. A simple cascade model that assumes top-down attention increases both the gain and the baseline BOLD activity in V1, V2/VP and V4 quantitatively explains the results. Our findings are consistent with single neuron recordings in monkeys and human visual event related potentials indicating that selective facilitation of neural responses to stimulation mainly occurs in extra-striate cortices.



Left: The profile of fMRI activity evoked by stimulation and attention. **Right**: The effect of cue timing on BOLD modulation (difference between pre- and post-cue conditions normalized to the average of the two conditions) varies systematically along the visual hierarchy.

80. Deployment of feature-based top-down attention during visual search

Ueli Rutishauser, Christof Koch

Where the eyes fixate during search is not random; rather, gaze reflects an expectation of where the subject expects the target to be. It is not clear, however, what information about a target is used to bias the underlying neuronal responses. We engaged subjects in a variety of simple visual search tasks while tracking their eye moments. We derive a generative model that reproduces these eye movements and calculate the conditional probabilities that observers fixate, given the target, on or near an item in the display sharing a specific feature with the target. We use these probabilities to infer which features were biased by top-down attention: color seems to be the dominant stimulus dimension-guiding search, followed by object size and, lastly, orientation. We use the number of fixations it took to find the target as a measure of task difficulty. We find that only a model that biases multiple feature dimensions in a hierarchical manner can account for the data. Contrary to common assumptions, memory plays almost no role in search performance. Our model can be fit to average data of multiple subjects or to individual subjects. Small variations of a few key parameters account well for the inter-subject differences. The model is compatible with neurophysiological findings of V4 and FEP neurons and predicts the gain modulation of these cells.

81. Is bottom-up attention useful for object recognition?

Ueli Rutishauser, Dirk Walther, Christof Koch, Pietro Perona[*]

A key problem in learning multiple objects from unlabeled images is that it is a priori impossible to tell which part of the image corresponds to each individual object, and which part is irrelevant clutter which is not associated to the objects. We investigate empirically to what extent pure bottom-up attention can extract useful information about the location, size and shape of objects from images and demonstrate how this information can be utilized to enable unsupervised learning of objects from unlabeled images. Our experiments demonstrate that the proposed approach to using bottom-up attention is indeed useful for a variety of applications.

[*]*Professor of Electrical Engineering, Caltech*

82. What do we perceive in a glance of a real-world scene?

Li FeiFei[1]*, Asha Iyer*[2]*, Christof Koch, Pietro Perona*[3]

What do we see when we glance at a natural scene and how does this visual information change as the glance becomes longer? We asked nave subjects to report in a freeform text what they saw in real-life photographs that were briefly presented to them. Our subjects received no specific information as to the content of each stimulus. Our paradigm differs from previous studies where subjects were either cued before a picture was presented, and/or were probed with multiple-choice questions. In the first stage, a group of 22 native English-speaking subjects were shown ninety novel grayscale photographs foveally. The presentation time was chosen at random in the set of seven possible times (from 27 msec to 500 msec). A perceptual mask followed each photograph immediately; subjects reported what they had just seen as completely as possible. In the second stage, another group of five naive individuals scored each of the descriptions produced by the subjects in the first stage. Individual scores were assigned to more than a hundred different attributes. We show that within a single glance, much object and scene level information is perceived by humans. But the richness of our perception is asymmetrical. Subjects have a bias to natural scenes being perceived as outdoor rather than indoor. The reporting of sensory or feature level information of a scene (such as shading, shape) consistently proceeds the reporting of the semantic level information. But once subjects recognize the more semantic level components of a scene, there is little evidence suggesting a bias toward scene level recognition over object level recognition, or vice versa.

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83. Spatial priors and task dominate the relation between attention and recognition

Wolfgang Einhäuser, Merrielle Spain[1], Pietro Perona[2]

In natural vision, humans sequentially attend to stimuli subsets for detailed serial processing. Nonetheless, observers can grasp the gist of a briefly presented scene without engaging attention. While recent studies demonstrate the usefulness of attentional models for machine vision in cluttered scenes, the details of the relation between attention and object recognition are not understood. Here we measure the relationship between object recognition and human attention in complex scenes, under natural conditions. We assess human attention by measuring observer's gaze direction while they view artistic photographs; we assess human recognition by immediate free recall of shown objects. To probe task modulation, some observers are additionally engaged in a visual search task during viewing. Using this setting, we demonstrate: Basic fixation parameters such as fixation duration, the spatial spread of fixated locations, and saccade length are significantly modulated by task. The objects recalled tend to be the objects fixated, with the largest fraction of fixations on the object recalled first. Part of the relation between fixation and recall is explained by a general, task-dependent but stimulus-independent, spatial prior on fixation. We observe a task modulated peak in fixation frequency near object boundaries, which is mostly attributable to boundary shape, length, and position. Our findings demonstrate that spatial priors and task dominate the relation between attention and object recognition.

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84. Graph-based visual saliency

Jonathan Harel[1], Pietro Perona[2], Christof Koch

Continuing in the now decades-old tradition [1], recent efforts have aimed at finding a fundamental quantity that is most highly correlated with human visual attention (see, e.g., "surprise" or "self-information"). Though attractive in principle, the computation of a reasonable interpretation of the quantity in practice typically requires a thoughtful algorithm with many sub-components. Here, we first organize the topology of these varied approaches, so that they can be compared more rigorously: i.e., not just end-to-end, but also piecewise, removing some uncertainty about the origin of observed performance differences. We then propose a new bottom-up saliency model, Graph-Based Visual Saliency (GBVS), which unifies the hitherto disparate problems of forming activation maps on certain feature channels, and then normalizing them in a way which highlights conspicuity and admits combination with other maps. The model is simple, and powerfully predicts human fixations on 749 variations of 108 natural images, achieving 98% of the ROC area of a human-based control, whereas the classical algorithms of Itti and Koch [2], [3], achieve only 84%. We find experimentally that there are at least two reasons for this observed difference. The first observation is that it is an emergent property that GBVS promotes higher saliency values in the center of the image plane. We hypothesize that this "center bias" is favorable with respect to predicting fixations due to human experience both with photographs, which are typically taken with a central subject, and with everyday life in which head motion often results in gazing straight ahead. We conjecture that the other reason for the performance difference stems from the robustness of our algorithm with respect to differences in the sizes of salient regions.

Our model is extensible to multi-resolution representations for better performance, is biologically plausible, and naturally lends itself to parallel implementation.

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[2]*Professor of Electrical Engineering, Caltech*

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Summary: Our field of specialization is neuroethology, which studies the neural mechanisms of natural behavior. When we plan to move from behavior to brain, we must decide what questions to ask and which brain area to study. The selection of brain areas is by no means easy, because it is hard to predict what neurons in a given area do with regard to specific behavioral tasks. Anatomical wiring diagrams do help make functional predictions under some circumstances. Although starting with primary sensory fibers would seem logical, it is hard to predict how the response properties of neurons change even in the second order station. The predictions here do not mean forecasting of all possible combinations or grouping of stimulus properties (such as frequency, amplitude, and time in the auditory system) that are encoded by the primary fibers but specific behaviorally meaningful configurations of the properties. What is the alternative then? We might start with "complex" neurons that are selective for behaviorally significant stimuli. The opinion that such neurons do not exist for complex stimuli derives from the implicit assumption that these neurons are isolated entities. Single neurons can be selective for complex stimuli such as faces. These "complex" neurons represent the results of many processes that occur in serial and parallel pathways leading to them. We must, however, assume that these neurons are tractable, i.e., anatomical connections and physiological processes leading to them can be determined. These pieces of information are not available for most of the complex neurons known today. The auditory system of barn owls is an exception among vertebrate sensory systems, because we have systematically investigated all stages of signal processing leading to the neurons that respond only to sounds arriving from specific directions in space.

In this academic year, two mathematically oriented members of our group, graduate student Bjorn Christianson and new postdoctoral fellow Brian Fischer, have carried out theoretical and computational analyses of neuronal responses relevant to sound localization. Bjorn obtained his Ph.D. in 2006 and is now a postdoctoral fellow at the University College, London, UK and Jose Luis Peña moved to the Albert Einstein College of Medicine as assistant professor. We wish their success.

85. Noise reduction of coincidence detector output by the inferior colliculus of the barn owl

G. Bjorn Christianson, Jose Luis Peña

A recurring theme in theoretical work is that integration over populations of similarly tuned neurons can reduce neural noise. However, there are relatively few demonstrations of an explicit noise reduction mechanism in a neural network. Here we demonstrate that the brainstem of the barn owl includes a stage of processing apparently devoted to increasing the signal-to-noise ratio in the encoding of the interaural time difference (ITD), one of two primary binaural cues used to compute the position of a sound source in space. In the barn owl, the ITD is processed in a dedicated neural pathway that terminates at the core of the inferior colliculus (ICcc). The actual locus of the computation of the ITD is before ICcc in the nucleus laminaris (NL), and ICcc receives no inputs carrying information that did not originate in NL. Unlike in NL, the rate-ITD functions of ICcc neurons require as little as a single stimulus presentation per ITD to show coherent ITD tuning. ICcc neurons also displayed a greater dynamic range with a maximal difference in ITD response rates approximately double that seen in NL. These results indicate that ICcc neurons perform a computation functionally analogous to averaging across a population of similarly tuned NL neurons.

86. Sound localization behavior of the barn owl is consistent with a Bayesian estimator

Brian Fischer

The barn owl can localize single sound sources with great accuracy in both the horizontal and vertical dimensions. When localizing sound sources, the barn owl must infer the direction of the source from the sound signals received at the ears. Behavioral experiments show that the barn owl uses the interaural time difference (ITD) for localization in the horizontal dimension and the interaural level difference (ILD) for localization in the vertical dimension. The nature of the inference problem that the owl faces depends on the relationship between locations in space and these binaural cues.

The spatial and frequency dependence of the localization cues ITD and ILD are revealed by making direct measurements of the sounds received at the barn owl's ears for sources in the environment. The qualitative picture of the localization cues derived from direct measurements is that ITD and ILD cues are quite complex functions of frequency that display spatial ambiguity. It is unclear what implications the spatial ambiguity of ITD and ILD cues have on operations that must be performed by the barn owl to support its localization of sound sources. Presently, theories suggest that a spectral matching operation underlies the derivation of spatial information from the auditory cues. However, there is yet to be a computational model that reproduces the sound localization behavior the barn owl.

Here we examined two models for the barn owl's sound localization behavior. We view the sound localization problem as a statistical estimation problem where direction estimates are derived from noisy measurements of spectral ITD and ILD cues. Maximum likelihood and Bayesian solutions to the estimation problem were compared with the localization behavior of a barn owl in a head turning task.

Our analysis supports the Bayesian model of the barn owl's solution to the localization problem over the maximum likelihood model. For noise variances large enough so that the maximum likelihood estimation error increased at the periphery, in accordance with the barn owl's behavior, the error also increased significantly in the central region of space. This non-monotonic shape to the maximum likelihood error with a large central peak is not consistent with the performance of the barn owl in the head turning task. In the Bayesian case, it was possible to find a Gaussian prior to produce a maximum a posteriori estimator with performance that matched the barn owl's behavioral performance. For the maximum a posteriori estimator, the error was smallest in the central region of space and increased at the periphery.

The maximum likelihood solution corresponds to a spectral cue matching operation, while the Bayesian solution uses a combination of spectral matching with a bias toward central locations. The performance of the estimators suggests that spectral matching models will not be sufficient to explain sound localization behavior in the barn owl and that an additional mechanism for resolving ambiguities in ITD and ILD must be considered.

The maximum likelihood model failed because of the nature of the spatial ambiguity in the ITD and ILD cues. As expected, directions near each other have similar cues. In addition to similarity of cues between proximal directions, distant directions can have similar ITD and ILD cues. The result of ambiguity between distant directions is that noise in measuring localization cues can lead to large errors in direction estimation, as seen in the maximum likelihood estimate. In particular, source directions in the central region of space are often mistaken for directions with similar horizontal position, but extreme peripheral vertical positions. While it is clear from behavioral tests that the barn owl does not confuse central locations with peripheral locations, there is not enough published data to determine if the owl confuses directions having peripheral elevations (elevation > 80 deg.) with directions in the center of space. Further experiments are required to test this prediction of the Bayesian model.

87. Combination of interaural time and level differences in ICcl

Brian Fischer

Space-specific neurons in the barn owl's space map derive their spatial selectivity from tuning to particular ranges of the interaural time difference (ITD) and the interaural level difference (ILD). Subthreshold responses of space-specific neurons suggest that the computation underlying the convergence of ITD and ILD is multiplication. This makes the barn owl's auditory system an ideal model system to study the generally important question of how neural systems perform multiplication. A prerequisite to studying the biophysical basis of multiplicative responses in space-specific neurons is to determine the nature of neural responses to ITD and ILD at the initial site of convergence of ITD and ILD in the lateral shell of the central nucleus of the inferior colliculus (ICcl). Towards this aim, we recorded extracellularly the spiking responses of single ICcl neurons to ITD and ILD in broadband noise signals presented over headphones.

Following Peña and Konishi [2001], ITD-ILD response matrices were fit with additive and multiplicative models of ITD-ILD interaction. The majority (84/89) of the ICcl spiking responses were better described by the multiplicative model than the additive model. While the multiplicative model better describes the data than does the additive model for most cells, there are properties of the neural responses to ITD and ILD that are not consistent with pure multiplication. ICcl neurons showed shifts of the preferred ITD with ILD, and vice versa, and displayed ITD tuning curves for different ILD that differed by an additive bias. These results show that the combination of ITD and ILD in ICcl is primarily nonlinear, but that responses are not as clearly multiplicative as in space-specific neurons.

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Summary: We are interested in information coding in the brain and in the design principles of circuits involved in processing sensory information. We are particularly interested in understanding the role of time, synchronization and oscillations in information coding and in relating the biophysical properties of neurons and synapses to the function of the networks in that they are embedded. We, therefore, study the cellular, synaptic and network aspects of neural processing. We focused our research this year on the olfactory system of insects (antennal lobes and mushroom bodies, circuits analogous to the vertebrate olfactory bulbs and anterior/posterior piriform cortices), using locusts, *Drosophila* and honeybees as primary model systems. Our work combines experimental (behavioral, electro-physiological and two photon imaging), and modeling techniques and aims at understanding functional aspects of brain circuits design and the rules of information coding used by the nervous system. Identity-specific responses with varying degrees of concentration invariance. The tuning of KCs to identity and concentration and the patterning of their responses are consistent with piecewise decoding of their PN inputs over oscillation-cycle length epochs.

88. Odor processing in the locust

Benjamin Rubin

The discovery of odor-induced oscillations in the locust brain* inspired a series of studies that have made the locust olfactory system a model for studying how information is represented and transformed across brain areas, and in particular for studying the role of neuronal oscillations in these processes. The majority of investigations of this system have focused on the ~800 projection neurons (PNs) of each antennal lobe (AL; the insect analogue of the vertebrate olfactory bulb) and their downstream targets, a population of ~50,000 Kenyon cells (KCs) located in the mushroom body (MB). These studies have provided considerable insight into how multiple factors (including the firing properties of PNs, PN-to-KC functional connectivity, cellular properties of KCs, and feed-forward inhibition of KCs) cooperate in transforming the highly dynamic and dense PN odor responses into specific and sparse KC responses.

Our ability to integrate our knowledge of this system, however, has been limited by three factors. First, no study has been made of how PN and KC populations respond to a wide variety of chemically similar and disparate odors – stimuli that have been instrumental in revealing olfactory coding principles in other animals. Second, investigations in which other stimulus parameters such as odor concentration, duration and mixtures have been varied are not directly comparable due to non-uniform experimental conditions. Third, since datasets across experiments cannot be combined, and no single experiment has sampled more than 150 PNs or KCs, questions that can only be addressed with larger datasets have remained unanswered. We plan to bridge these gaps by producing an extensive dataset of many hundreds of PNs and KCs recorded under uniform conditions in response to widely varying odor stimuli. To this end, we have designed and built a computer-controlled odor delivery system capable of delivering up to 169 odors and are in the midst of constructing the aforementioned physiological dataset (Figure 1). While the overarching goal of this work is to produce a model that codifies and challenges our understanding of the system, this study will allow us to address specific coding questions while creating a dataset that will be used to answer additional questions that will undoubtedly arise along the way.

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Figure 1. An example of the responses of a single PN to a diverse odor set. 21 odor blocks are shown, each odor having been delivered seven times (20 s trials, 1 s odor stimuli indicated by the shaded bars). Tick marks represent single extracellularly recorded action potentials. Virtually every odor in this set evoked a distinct response. The figure represents 49 continuous minutes of this PN's activity, starting from the top left (odor: 1-hexen-3-ol), proceeding downward, and ending at the bottom right (empty odor vial). Other odors presented include primary alcohols, unsaturated alcohols, cyclic terpenoids, and ethers.

89. Odor representations in the *Drosophila* mushroom body

Glenn C. Turner, Gilles Laurent

The mushroom body (MB) of the insect brain is essential for olfactory learning and memory. We are using electrophysiological techniques to study odor representations in the MB of *Drosophila*, an insect with established olfactory conditioning paradigms and genetic variants with specific learning and memory defects. Our goal is to determine how odors are represented in a brain area involved in learning. We have used whole cell patch clamp recordings to characterize the odor response properties of the Kenyon cells (KCs), the principal neurons of the MB. All recorded KCs were spiking neurons and fired sodium action potentials in response to current injection. In the absence of odor stimulation KCs showed significant ongoing synaptic activity. Baseline firing rates, however, were extremely low. Odor presentation increased subthreshold synaptic activity, but the overall probability of a spiking response to a given odor was only ~5%. Thus, KCs are highly odor selective compared to their inputs, the antennal lobe (AL) projection neurons, which have a 50% probability of response (1). A similar transformation from dense representations in the AL to sparse representations in the MB was first observed in locust (2). A sparse format minimizes overlap between the representations of similar stimuli and could contribute to the accuracy of memory formation and recall. We are currently examining the mechanisms responsible for this transformation. Although periodic phase-lagged excitatory-inhibitory synaptic inputs to KCs are essential for sparse representations in locust, we do not find strong evidence for such oscillations in *Drosophila*. We are examining the contribution of other mechanisms including: i) fast synaptic kinetics; ii) spike threshold and, iii) phasic, non-oscillatory inhibition.

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90. Synaptic transfer function in *C. elegans*

Anusha Narayan, Gilles Laurent, Paul Sternberg[*]

The neural circuits of the nematode *C. elegans* have been characterized based on anatomical connectivity. There is currently very little known about the dynamic functioning of these cells, and, more fundamentally, how information is transmitted across a *C. elegans* synapse. We intend to characterize a synaptic transfer function in *C. elegans*, to explore how the dynamic range of the post-synaptic response is set, and to understand the mechanisms for integration and gain control in this system.

In order to address these questions, one needs a controlled, precise way of stimulating the presynaptic cell. Channelrhodopsin (ChR2), a light activated cation channel has been used to obtain genetically targeted optical control of neural activity, with a timescale of milliseconds [1]. In *C. elegans*, light-activation of neurons and muscles expressing ChR2 has been shown to evoke behavioral responses [2]. Our plan is to use cell-specific promoters to express ChR2 in the presynaptic cell and gfp in the postsynaptic cell, and monitor cellular activity using whole-cell patch clamp.

We selected the AFD-AIY synapse based on criteria including the following: A demonstrated behavioral relevance of the synapse, an absence of feedforward connections and direct feedback, and the presence of unique cell-specific promoters. AFD is a thermosensory neuron that measures the difference between ambient temperature and preferred temperature (temperature of cultivation). Imaging studies from AFD [3] indicate that AFD can phase-lock its calcium dynamics to oscillatory thermosensory inputs. AIY is an interneuron that is the primary postsynaptic partner of AFD. It also receives input from chemosensory neurons ASE, AWA and AWC. AIY is thought to mediate locomotor responses to chemosensory and thermal stimuli transduced though AFD, ASE and AWA [4].

Since ChR2 allows millisecond-timescale, targeted control of stimulus, with electrophysiology it should be possible to obtain temporally precise information about the dynamics of the synaptic transfer function between AFD and AIY. This is the first step towards understanding the functional dynamics of neural sub-circuits in *C. elegans.*

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91. Encoding and decoding of overlapping odor sequences

Bede M. Broome[*]*, Vivek Jayaraman*[*]

Odors evoke complex responses in locust antennal lobe projection neurons (PNs)—the mitral cell analogs. These patterns evolve over hundreds of milliseconds and contain information about odor identity and concentration. In nature, animals often encounter many odorants in short temporal succession. We explored the effects of such conditions by presenting two different odors with variable intervening delays. PN-ensemble representations tracked stimulus changes and, in some delay conditions, reached states that corresponded neither to the representation of either odor alone, nor to the binary mixture of the two. We then recorded from Kenyon cells (KCs), the PNs' targets. Their responses were consistent with the PN population's behavior: in some conditions, KCs were recruited that did not fire during single-odor or mixture stimuli. Thus, PN-population dynamics are history dependent, and responses of individual KCs are consistent with piecewise temporal decoding of PN output over large sections of the PN population.

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Publication

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92. Simultaneous electrophysiology and two-photon imaging of olfactory projection neurons in intact fruit flies

Vivek Jayaraman

Genetically encoded optical indicators hold the promise of enabling non-invasive monitoring of activity in identified neurons in a behaving organism. The interpretation of images of brain activity produced using such sensors is, however, problematic, and may be the source of seemingly contradictory findings in *Drosophila* olfaction. At issue is the breadth of odor tuning of projection neurons (PNs), the output neurons of the antennal lobe (the insect analog of the mammalian olfactory bulb). One group performed 2-photon imaging experiments in detached antennae-brain preparations by expressing a calcium-sensitive fluorescent protein named G-CaMP[1] in PNs. They interpreted the selective G-CaMP responses they recorded to mean that PNs are rather narrowly tuned to odors, and suggested that a given PN's responses directly reflect the uniglomerular input it receives from its olfactory sensory neurons (OSNs)[2]. This is in contrast to evidence from *in vivo* whole-cell recordings suggesting that PNs are more broadly tuned, consistent with a transformation of OSN input by the antennal lobe circuitry[3]. A different group using G-CaMP expressed in Kenyon cells, targets of the PNs, has suggested that odors evoke sparse, stereotyped and spatially restricted responses in these neurons[4], a finding that is consistent with electrophysiological data[5]. In both sets of imaging experiments however, it is unclear what electrophysiological signal the imaging signal being measured corresponds to. The narrow odor tuning seen in these imaging experiments could, for example, be a result of the relatively high threshold of activation of G-CaMP[6,7].

We are attempting to resolve such issues by performing simultaneous 2-photon imaging and electrophysiology of G-CaMP expressing PNs in an intact, *in vivo* adult fly preparation. For our electrophysiological recordings in PNs, we use both loose-patch[8] and intracellular (sharp electrode) techniques. In our current experiments, we are trying to establish the correlation between physiology and G-CaMP signal in PN somata. Imaging in the soma, we find that G-CaMP faithfully reports sustained (e.g., >1 sec) activity at rates higher than 50Hz, albeit with very slow kinetics. However, it is unable to detect sustained activity at rates below 30Hz or higher-rate activity if it is not sustained (e.g., 50Hz for 200 m sec). Our results suggest that while this sensor can be useful for the identification of neural targets for more refined exploration of a functional circuit, it could be misleading if used as the sole means to assess activity in a population of neurons.

More generally, the methodology we have developed enables any genetically encoded sensors, activators or silencers to be precisely calibrated in an intact fly and in the very neurons that they are likely to be used in, something that is crucial given the likely cell-type-dependent variability of their function. Calibrated use of such fast-improving technology is likely to be of considerable value to systems neuroscience.

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93. Functional organization of the central complex in an insect brain

Cindy Chiu

The central complex is a midline neuropil brain structure that is present in most insects. Its most striking feature is a three-dimensional matrix structure which interconnects the left and right hemispheres through an ordered set of chiasmata.[1,2]. Although the intrinsic anatomy of the central complex is well-mapped, the input-output connectivity of this region is poorly understood. Based on behavioral studies of *Drosophila melanogaster* central complex mutants and a small number of electrophysiology recordings in locusts, the central complex has been implicated in "higher order" brain functions including integration of visual sensory information and regulation of motor-related activities.[3,4,5] Additionally, its systematic inter-hemispheric connectivity points to a role in mapping external space and integrating this information across the left and right hemispheres. Despite these tantalizing possibilities, the essential function of the central complex remains elusive.

My goal is to elucidate the functional organization of the central complex. To date, there are few electrophysiological data of central complex neurons. Given the broad range of possible inputs to this structure, I have chosen a battery of diverse, ethologically relevant stimuli. Currently, I am assembling an electrophysiology rig that includes an arena of visual, auditory, olfactory, and mechanosensory stimuli. Using this broad-based stimulus repertoire, I aim to: (1) Characterize using intracellular methods the electrophysiological and anatomical properties of individual central complex neurons; (2) examine the physiological responses of central complex neurons during orientation behaviors and, (3) investigate the circuit properties of the central complex system by Precording from neural ensembles. I have chosen for my model organism the field cricket, *Gryllus bimaculatus*, because it is amenable to *in vivo* intracellular and extracellular electrophysiology methods, and it exhibits robust behaviors that can be studied in laboratory conditions.

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947. Olfactory representations in the *Drosophila* mushroom body

Mala Murthy

Over the past year, I have investigated the logic of neuronal connectivity in the *Drosophila* mushroom body, a higher order brain center required for complex processes such as learning and memory. The mushroom body (MB) is important for olfactory learning: Molecules implicated in learning are concentrated in the MB, mutants that disrupt these molecules have defects in memory formation, and anatomical studies have shown that experience can induce substantial structural transformations. Such plasticity suggests that odor representations by Kenyon cells (KCs), the neurons that comprise the MB, by their targets, or both, could change within the lifetime of each animal or across animals. Further, KCs belong to large populations (2,500 in *Drosophila*; 300,000 in cockroaches; millions in the related arthropod *Limulus*) of similar neurons: morphological and molecular subtypes have been described, but identifiable individuals have not. Yet, the neurons from which they receive olfactory input, the projection neurons (PNs), are unambiguously identifiable, by virtue of their restricted projections to individual antennal lobe glomeruli, expression of molecular markers, and odor responses. In the *Drosophila* MB, there is conflicting evidence regarding anatomical and functional stereotypy, and it is not known if the matrix of connections between PNs and KCs is predetermined, thus giving rise to stereotyped odor responses within identifiable KCs, or if this matrix of connections is more random, thus varying across individuals. The extent to which KCs are deterministically stereotyped affects how experience and learning may modify odor representations within the MB. To address this question of stereotypy, I have been conducting *in vivo* whole-cell patch-clamp recordings from a subpopulation of labeled KCs in *Drosophila melanogaster*, in order to record from the same cell or cells across genetically identical flies. Answering this question with KCs will inform what could be expected with larger systems of neurons in other organisms.

In the fruit fly, the mushroom body is derived from four neuroblasts, and by divisions through the embryonic, larval and pupal stages, each neuroblast generates ~500 neurons per clonal unit. Each cluster can be subdivided into three KC classes (α/β, , and α'/β') based on adult axonal projection patterns and birth order. The *Drosophila* GAL4/UAS system allows for spatial control over transgene expression, based on the promoter regulating the GAL4 insert. Many labs have identified GAL4 lines with expression in the MB, and I have screened through 60 such GAL4 lines for those with very restricted KC expression. GAL4 line *NP7175* is expressed in a small subset of KCs (23.6±3.3 KCs per neuroblast clonal unit; 20 clonal units counted) that project to the core of the α/β lobes. Moreover, each of the four clonal units can be distinguished based on the position of somata and the region of the MB calyx innervated by their dendrites, which allows for identification of the same ~23 cells across flies. In order to sample at least three duplicate

KCs, I have recorded from 18 KCs from the left hemisphere, lateral posterior (L, LP) clonal unit of *NP7175 GAL4; UAS eGFP2x/Cyo* 0-1 day old females, raised under identical conditions. Even assuming that there are as many as 30 cells per clonal unit (the maximum number of cells counted for an *NP7175* clonal unit), 18 recordings results in >99% probability of sampling at least two cells twice. I have studied the odor tuning of KCs stimulated with 43 odors (12 odors at three concentrations, plus control odors) and have confirmed cell identity with intracellular labeling. Because neuronal responses in KCs are sparse, that is responses are elicited by only a small percentage of odors, KCs can easily be identified both by their spiking and subthreshold activity in response to odors. For comparison, I have also recorded from 7 GFP-labeled KCs from other clonal units and 15 non-GFP-labeled KCs, whose cell bodies are adjacent to L, LP *NP7175* KCs, but whose axons project to disparate regions of the MB lobes.

I have begun to characterize KCs based on the following four criteria: 1) The morphology of axon branches, reconstructed from biocytin fills; 2) the presence of spikes during the odor response period, and the rate and timing of spikes if present; 3) subthreshold responses during the odor response period (i.e., dynamics of membrane depolarization); and, 4) rank orders of response strength to the odors tested. Thus far, I do not find evidence for replicates among cells from the same clonal unit, suggesting that KCs are not stereotyped. The preliminary implication of these findings is either that individual KCs connect with a different subset of PNs in every fly or that synaptic weights are set differently among a constant matrix of PN to KC connections.

To follow up these results, I am currently investigating the possibility that *NP7175* KCs from the L, LP clonal unit, while not stereotyped, may possess odor response profiles that are more similar to one another than to KCs that project to other regions of the MB. In addition, it is possible that while NP7175 KCs are not stereotyped, other KCs are, and for this reason, I have identified a second GAL4 driver that labels a distinct small subset of KCs, to determine if KCs that project to other regions of the MB lobes are similarly lacking in response stereotypy. Finally, I have conducted similar recordings on identifiable projection neurons across many flies. I have recorded from PNs that innervate antennal lobe glomerulus DL1, in response to a panel of approximately 12 odors. Because PNs have been shown to be stereotyped, this control will serve to validate my methods of analysis on the KCs.

While a lack of stereotypy in KCs is interesting, an absence of a functional map in the MB would make it nearly impossible to undertake experiments focused on long-term plasticity and learning in KCs. I have, therefore, begun to investigate neurons that are postsynaptic to the KCs, the MB extrinsic neurons. In this vein, we have generated several fly lines that label MB extrinsic neurons with eGFP and with GCaMP, a genetically-encoded calcium sensor. Future research will be focused on characterizing odor responses in these cells, and determining how experience can modify these responses. In addition to my results on stereotypy, I have observed, consistent with results from the Laurent lab in the locust, that some KCs are concentration selective, while other KCs are odor selective, but respond to all concentrations tested. Behavioral experiments in flies have shown that valence is correlated with concentration for many odors, and it will be of interest to determine how MB extrinsic neurons read out this concentration specific information. Ultimately, it will be important to screen for GAL4 lines that label neurons postsynaptic to the extrinsic neurons, to understand how sensory perception results in behavioral choices for the fly.

95. Plasticity of olfactory representations in zebrafish larvae

Jonathan Young

We are interested in the question of olfactory circuit plasticity: Does the representation of an odor object change over the life history of the animal, and what impact does this have on olfactory computation? The zebrafish larva provides a vertebrate model system for studying olfaction that offers the advantages of complete transparency, complex behavior, established genetics, and external development, which are the properties needed to investigate this question. Work has already previously been done in this lab to characterize the responses of mitral cells in the adult zebrafish olfactory bulb. We will use the larval form of the zebrafish to study olfactory learning and circuit development.

Using a combination of behavioral, electrophysiological, and calcium imaging techniques, we will investigate the capabilities of the larval olfactory system to learn and change through development, and observe how these changes are encoded in both the activity of the olfactory bulb and the behavioral responses of the animal. We will aversively condition fish to odors while simultaneously either imaging calcium activity in the olfactory bulb using two photon microscopy or doing electrophysiological recording from mitral cells. We hope to be able to observe changes in the activity of the mitral cell population of the olfactory bulb during olfactory learning and development, and gain an understanding of the circuit mechanisms underlying these processes.

96. Processing of multisensory information in the honeybee central complex

Mikko Vähäsöyrinki

The success of an individual depends upon accurately determining and executing the appropriate behavior based on sensory information gathered from the surroundings. How does the nervous system extract biologically relevant information from a complex multisensory environment to generate adaptive behaviors? To address this question my research is focused on studying the neuronal representation of olfactory and visual information in the honeybee's (*Apis mellifera M.*) central complex.

The central complex (CX) is a prominent, bilateral, invariable structure in the center of insect brain. Although earlier studies have demonstrated its role in higher motor control and visually guided behavior[1,2], functions of this brain area are poorly understood and the corresponding neuronal mechanisms largely unknown. The CX comprises two major subdivisions: The protocerebral bridge and the central body, which can itself be further subdivided into an upper and lower division and a pair of globular noduli. Interestingly, the CX is organized topographically, with horizontal layers intersected by vertical columns (Figure 1a). This organization, together with its central location in the brain, suggests some involvement in the bilateral integration of signals across both hemispheres and provides an intriguing model system for studying information processing in neural networks.

I have developed an experimental setup and an *in vivo* preparation that allow electrophysiological recordings while stimulating bees with visual and olfactory stimuli. My current focus is to establish a basic functional characterization of main types of central complex neurons by doing intracellular recordings with fluorescent dye injections (Figure 2). This information will also form the basis for cell-type identification from extracellular multiple single–unit recordings with tetrodes. These experiments are aimed at providing a network-level description of multisensory processing in the honeybee central complex.

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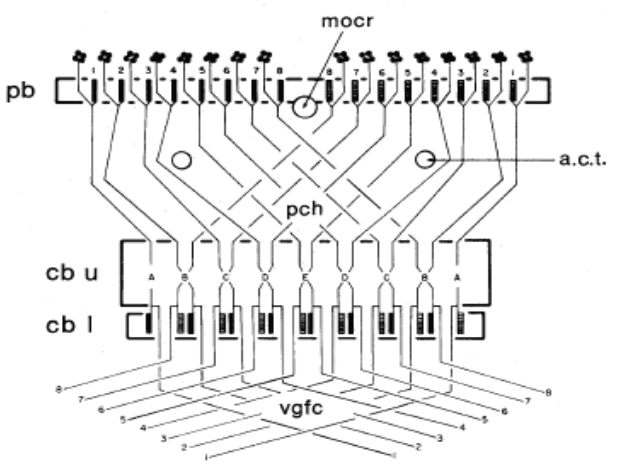

Figure 1. Diagram showing the arrangement of 64 neurons in the central complex of *Schistocerca gregaria*. Taken from [1].

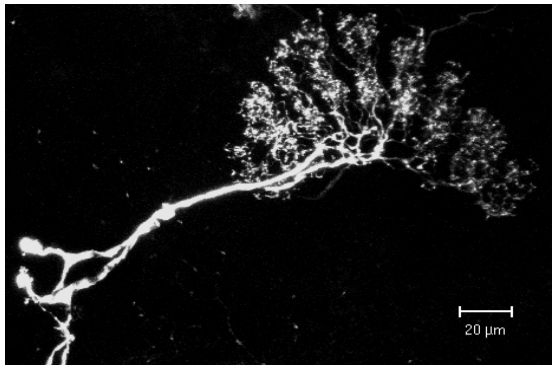

Figure 2. An example of two central body lower unit neurons (honey bee).

97. Decoding the sparse representations of the locust mushroom body

Stijn Cassenaer

The insect mushroom body (MB) is used as a model system to address several questions of general neurobiological interest such as sensory discrimination, multi-modal integration, the control of complex behavioral repertoires, as well as learning and memory[1]. Among these, the role of the MB in olfaction and memory has received considerable attention. A number of studies have addressed how olfactory information reaches the MB and how it is transformed along the way[2,3]. A particularly dramatic transformation occurs between the antennal lobe (AL) and the MB. Whereas, odor representations in the AL are densely distributed and dynamic, only one synapse further downstream, odor representations are sparse, comprised of brief activation of a small fraction of the MB intrinsic neurons (Kenyon cells, KCs). This sparse representation should be advantageous for learning and memory, as it reduces the number of synapses that need to be modified and the number of comparisons necessary for pattern matching[4]. There are several lines of evidence that implicate the synaptic contacts between KCs and their downstream targets in memory storage and recall[5].

In order to address the question of how the sparse representations carried by KCs are decoded and further transformed before leaving the MB, we are investigating the cells that take KC activity as their input, the MB extrinsic cells. There appear to be several distinct cell types within this population that differ most obviously with respect to the neuropils they invade[6,7]. We are characterizing these neurons in terms of their physiological and morphological properties by means of intracellular and tetrode recordings, as well as extracellular stimulation and pressure-microinjection. We are investigating how odors are represented by this population, how efficacious the KC-extrinsic cell synapses are, how they can be modified, and how the extrinsic cells integrate their KC inputs.

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98. Role of inhibition of the giant GABAergic neuron in the mushroom body

Maria Papadopoulou, Glenn Turner

The giant GABAergic neuron (GGN) is a non-spiking neuron that arborizes extensively in the mushroom body (MB) to contact the dendrites of Kenyon cells (KCs) (Leitch and Laurent, 1996), neurons required for learning and memory (Heisenberg, 2000). We are interested in characterizing the functional role of GGN in the olfactory circuit because its pattern of connectivity makes it an attractive candidate for modulating KC responses with potential implications for learning and memory. Physiological recordings of KCs in locust have shown these cells to represent odors sparsely despite receiving continuous, dense and slowly evolving excitatory input from antennal lobe projection neurons (Perez-Orive *et al.*, 2002). Inhibition appears to be important for the sparseness and brevity of the KC response, because it contributes to odor-induced oscillations of the KC membrane potential that create a narrow window of KC temporal integration of excitatory input (Perez-Orive *et al*, 2002). While part of the source of inhibition has been identified, we are interested in elucidating the role of the odor-evoked GGN inhibition in the sparseness and synchrony of KC responses. More specifically, the goal is to determine the role of the graded aspect of the odor-evoked GGN response to increasing odor concentration and its functional consequences for the KC population response. This work could have implications for learning and memory since sparseness in the KCs has been suggested as a mechanism to facilitate storing and handling of memories (Perez-Orive *et al*., 2002).

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99. Population coding of odor mixtures of increasing complexity by principal neurons of the antennal lobe

Kai Shen, Gilles Laurent

In nature, animals rarely encounter stimuli in isolation and must often extract meaningful information from complex odor mixtures. Understanding how the brain treats such complex stimuli is complicated by the observation that olfaction is a synthetic sense. Unlike vision, where we can easily parse out the individual lines in a Kadinsky painting, in olfaction, we can identify individual components in a mixture, but only if less than three-four odors are mixed together. How then, does the brain code odor mixtures?

I address this question using electrophysiological methods. Currently, I am examining how the Antennal lobe and Mushroom body (first and second relay in the locust brain) code odor mixtures. Experiments involve inserting electrodes into the respective brain regions of the locust and recording the electrical activity (extra-cellular spikes) of neurons in those regions, while presenting odor mixtures of increasing degrees of complexity to the animal. By looking for structure and pattern in the resulting high dimensional data set (consisting of the spiking activity of many neurons), I hope to uncover the underlying circuit dynamics of the system.

We recorded the single-unit activity of ensembles of projection neurons (PNs), the principal cells of the antennal lobe, and Kenyon cells (KCs), the intrinsic cells of the mushroom body, as well as the local field potential (LFP) within the mushroom body, while presenting mixtures of monomolecular odors. First, we examined the evolution of PN ensemble response patterns as we systematically varied the ratio of concentrations of two components within a binary mixture. Applying principal component analysis for dimensionality reduction of the PN population response, we observed that responses to different mixtures formed distinct clusters and varied in a nonlinear manner with odor ratio. We applied locally linear embedding to visualize the time-varying dynamics of the PN ensemble as a function of the ratio of odor components. Second, we estimated the ensemble PN response vectors to odor mixtures by adding the response vectors corresponding to the mixture components. We then examined the degree of deviation between these estimated response vectors and the experimentally observed response vectors as the number of odorants within the mixture increased from 2 to 3, 4, 5, and 8. Finally, we examine the degree to which these representations mirror the responses of the targets of the PNs in the mushroom body, the KCs. This will give us insights into what exactly happens in the brain when we encounter a very complicated odor mixture.

100. Stimulus tuned criticality, short-term memory and optimized computation in the olfactory system

Ingmar Riedel Kruse

I want to understand how neuronal networks can learn to optimally perform computational tasks based on their network topology and neuronal dynamics - a central question in systems-neurobiology.

I will study the antenna lobe (AL) in the locust olfactory system - a neuronal network carrying out highly dynamic odor discrimination tasks. Repeated odor encounters tune the neurons in the AL towards an odor specific synchronous oscillatory firing pattern. This tuning is a form of unsupervised learning (or short-term memory). Transient spontaneous oscillations are still present when the odor stimulus is absent, which strongly suggests to me that this system is close to criticality.

Systems close to criticality show strong non-linear behavior and have, therefore, been proposed to be optimal sensors with high sensitivity and large dynamic range. One of the rare biological examples with convincing evidence for this proposition is hearing, were the self-tuning mechanism of the hair-bundle is close to an oscillatory instability.

In abstract analogy to hearing I will: (1) experimentally test whether the AL truly utilizes criticality; (2) experimentally investigate how criticality facilitates odor discrimination and memory formation; and (3) develop a theoretical model to understand advantages and limitations of criticality in the AL.

I will learn and apply single and multi-neuronal recording techniques and combine them with my experimental and theoretical expertise gained from working on the dynamics of other complex biological systems outside neurobiology. My work aims for conceptual new insight into olfactory processing, memory formation and the role of the widely observed oscillatory activity in neuronal networks.

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Summary: We continue our work on biophysical, neuroscience, and medical aspects of ion channels, receptors, and transporters. We have analyzed several strains of knock-in mice generated in our laboratory for the nicotinic α4 receptor, and these have resulted in interesting insights into nicotine addiction. We find that activation of α4-containing receptors by nicotine is sufficient for tolerance, sensitization, and reward behavior. The nicotinic receptor work is enhanced by a promising new knock-in strain, with fluorescent α4 receptors. We are beginning to understand the nicotine-dependent, cell type-specific increase in receptor levels, and how this increase produces some aspects of nicotine addiction.

The group's research has been enhanced during the past year by two new microscopes: a Nikon spectrally resolved confocal system, and TIRF capabilities for an existing wide-field instrument. The TIRF capabilities are being extended to single molecule resolution.

The α4 nicotinic receptor, or its partner, the β2 subunit, are mutated in a rare series of human epilepsies. We studied the nAChR epilepsy mutations in oocyte expression systems and, in collaboration with other labs, are constructing knock-in mouse strains that also have these mutations. Another α4 knock-in strain, the Leu9'Ser, presents a model system for studying neurodegenerative disease.

Our work on selective silencing of mammalian neurons has generated a promising set of techniques and reagents based on ligand-activated chloride channels. In collaboration with the David Anderson laboratory, we are now completing the initial experiments on "proof of concept" silencing in mice; and with the group of Herwig Baier at the University of California, San Francisco, to zebrafish.

We continue our joint work with the Dougherty group, in Caltech's Chemistry Division, on aspects of ion channel structure function. We have brought novel techniques to these studies, including mass spectrometry and fluorescence. We also concentrate on unnatural amino-acid mutagenesis. We have now found that a cation-π interaction helps agonists bind to several cys-loop receptors—for acetylcholine, serotonin, and GABA. By employing the new TIRF microscope, we are measuring individual nicotinic receptors containing unnatural florescent side-chains.

With the Dougherty group and with Neurion Pharmaceuticals in Pasadena, we have started to analyze the binding of blocking drugs to the hERG potassium channel, also using unnatural amino acid mutagenesis. The channel is important because it underlies many instances of drug-induced long-QT syndrome. We are working hard to incorporate unnatural amino acids into the hERG channel when expressed in mammalian cells, where the pharmacology of blockade is much more similar to that in heart muscle.

Our work continues on quantitative aspects of transporter function, primarily measured with fluorescence and with knock-in mice. We are using fluorescence to analyze the mobility of GABA transporters, and both confocal and TIRF modes. As an interesting side benefit of the GABA transporter knock-in mouse, we have generated and analyzed a knockout mouse for the same molecule.

Our group's home page has additional up-to-date information, images, and notices of positions. It's at http://www.cco.caltech.edu/~lester.

101. The role of cation-π interactions in nicotinic receptor activation

Jai A.P. Shanata, Bruce N. Cohen, Dennis A. Dougherty*, Henry A. Lester*

Our previous results show that a cation-π interaction between the agonist acetylcholine (ACh) and a tryptophan residue (αW149) in the extracellular domain of the muscle nicotinic acetylcholine receptor (nAChR) dramatically enhances the ability of ACh to activate this receptor. To activate a receptor, the agonist must first bind to it and then induce the conformational change(s) leading to activation. To determine which of these steps the cation-π interaction at residue αW149 primarily affects, we expressed wild-type (WT) and mono-fluorinated mutant (5-F-W149) nAChRs in *Xenopus* oocytes and recorded their single-channel activity in cell-attached patches with 300 μM ACh. We previously found that modifying the cation-π interaction at residue αW149 by fluorinating this residue (5-F-W149) reduces the ACh sensitivity (i.e., increases the EC_{50}) of the muscle nAChR six-fold. Analyses of our single-channel kinetic data show this mutation reduced the ACh gating equilibrium constant ( ) less than twofold, far short of the 36-fold decrease required to account for the six-fold increase in EC_{50} ($EC_{50} \sim {}^{-1/2}$). In contrast, fits of the WT and 5-F-W149 mutant single-channel data to a four-state kinetic model with three agonist-bound closed states suggest that the 5-F-W149 mutant reduced the apparent forward rate constant for Ach-binding seven-fold, enough to account for the effects of tryptophan fluorination on the EC_{50}, but this rate constant could also reflect rapid desensitization of the receptor rather than agonist binding. Thus, our single-channel data suggest that the cation-π interaction between ACh and residue αW149 in the muscle nAChR probably affects ACh binding rather the ACh-induced conformational changes leading to receptor activation. Yet we cannot exclude the possibility that it may be involved in receptor desensitization. Future work will include studies of the di-fluorinated (5,7-F_2-W149) and tri-fluorinated mutants (5,6,7-F_3-W149), and the effects of other ACh concentrations on the kinetics of the mono-fluorinated 5-F-W149 mutant. Also, we will confirm that the cation-π interaction at αW149 does not affect the s measured for other agonists.

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102. *In vivo* incorporation of multiple unnatural amino acids through nonsense and frameshift suppression

Erik A. Rodriguez, Henry A. Lester, Dennis A. Dougherty**

Site-specific incorporation of unnatural amino acids (UAAs) into proteins is a valuable tool for studying structure–function relationships, incorporating biophysical probes, and elucidating protein–protein interactions. In higher eukaryotic cells, the methodology is currently limited to incorporation of a single UAA in response to a stop codon, which is known as nonsense suppression. Frameshift suppression is a unique methodology for incorporating UAAs in response to quadruplet codons, but currently, it is mostly limited to *in vitro* protein translation systems. Here, we evaluate the viability of frameshift suppression in *Xenopus* oocytes. We demonstrate UAA incorporation by using yeast phenylalanine frameshift suppressor (YFFS) tRNAs that recognize two different quadruplet codons (CGGG and GGGU) *in vivo*. Suppression efficiency of the YFFS tRNAs increases nonlinearly with the amount of injected tRNA, suggesting a significant competition with endogenous, triplet-recognizing tRNA. Both frameshift suppressor tRNAs are less efficient than the amber suppressor tRNA THG73 (*Tetrahymena thermophila* G73), which has been used extensively for UAA incorporation in *Xenopus* oocytes. However, the two YFFS tRNAs are more "orthogonal" to the *Xenopus* system than THG73, and they offer a viable replacement when suppressing at promiscuous sites. To illustrate the potential of combining nonsense and frameshift suppression, we have site specifically incorporated two and three UAAs simultaneously into a neuroreceptor expressed *in vivo*.

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103. Probing the importance of backbone interactions in the nAChR using α-hydroxy acids

Kristin D. Rule, Henry A. Lester, Dennis A. Dougherty**

The importance of backbone hydrogen bonding and flexibility in the extracellular domain of the nicotinic acetylcholine receptor (nAChR) were explored using unnatural amino acid mutagenesis. Incorporating an α-hydroxy analogue of an amino acid replaces the backbone amide bond with an ester. This subtle mutation disrupts hydrogen bonding and confers additional backbone flexibility at the site of mutation without perturbing the side chain. While amide-to-ester mutations of the binding box residues in the primary subunit (α) produced only modest changes in receptor function, they pointed to an important role for residues in key β-strands near the binding site. Anomalous behavior was observed for α-hydroxy acids incorporated at the binding-site residues in the complementary subunits (and) that suggests a key role for these residues in nAChR function.

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104. The functional role of a conserved aspartate in the nicotinic acetylcholine receptor

Amanda L. Cashin, Michael M. Torrice*, Kathryn A. McMenimen*, Henry A. Lester, Dennis A. Dougherty**

The nicotinic acetylcholine receptor and related Cys-loop receptors are ligand-gated ion channels that mediate fast synaptic transmission throughout the central and peripheral nervous system. A highly conserved aspartate residue (D89) that is near, but not integral to, the agonist-binding site has been proposed to play a critical part in receptor function. We have probed the role of D89

with the unsurpassed precision of unnatural amino acid mutagenesis coupled with electrophysiology. The side chain of D89 establishes a redundant network of hydrogen bonds that serves to preoganize the agonist-binding site by positioning a critical tryptophan residue that directly contributes to the agonist-binding site. The negative charge of D89 is not essential for receptor function. Our studies also revealed a novel electrostatic clash in the highly deleterious D89N mutant that rationalizes the behavior of a unique Cys-loop receptor, the MOD-1 serotonin receptor, which naturally contains this substitution.

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105. Contrasting drug-receptor interactions at neuronal vs. muscle-type nicotinic acetylcholine receptors: The 4 4 neuronal receptor

Xianan Xiu[1], Mark Nowak[2], Niki Zacharias[2], S. Richard Baker[3], Michael Bodkin[3], Nima Shiva[2], Emanuele Sher[3], Dennis A. Dougherty[1]

This study begins to apply the nonsense suppression approach to unnatural amino acid incorporation for nicotinic receptors (nAChR) expressed in *Xenopu*s oocytes. A number of unnatural amino acids were incorporated and evaluated in the 4 4 nAChR. Remarkably, the compelling cation-π interaction seen between ACh and TrpB of the muscle-type receptor is mostly absent in the 4 4 receptor. None of the other four side chains probed in the aromatic-binding box shows strong evidence for an equally strong cation-π interaction with ACh. However, a modestly large EC50 ratio of ~15 was observed for the CN vs. Br Phe derivatives in loop A, consistent with weak cation-π interaction. We also evaluated three other agonists - nicotine, epibatidine, and cytisine. At TrpB, for nicotine to some extent and definitely for cytisine, 5-CN-Trp causes a much large drop in potency than does 5-Br-Trp, possibly indicating a cation-π interaction. Within the nAChR family, cation-π interactions appear to vary markedly, despite high homology and absolute identity for the supposedly key residues.

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106. Fluorescence and crosslinking studies of the muscle nicotinic receptors

Mohammed Dibas, Rigo Pantoja

We continue to use fluorescence to study conformational transitions at the muscle nAChR. We tethered a sulforhodamine fluorophore to M2 19' position in the upper M2 helix of either , , , ε, or δ subunit. Good fluorescence signals (ΔF) can be obtained from the MTSR-labeled M2-Cys19' position of any nAChR subunit during activation. Yet each subunit has a characteristic amplitude and sign of ΔF. Interestingly, the ΔF for either the γ, or its paralog the ε subunit, is negative, as opposed to the pattern for all the other subunits the oocytes were injected with αβ(γ19'Cys)δ or αβ(ε19'Cys) δ, respectively. We have combined these data with signals obtains from mutations at the M2 16' position and at neighboring positions in the M1 and M3 helices. We have also assessed Cu phenanthroline crosslinking of dual Cys mutations. The data help to define the contacts of the M2 19' position in the resting and active states.

107. Microorganization of synaptic receptors

Lawrence A. Wade

Nicotinic acetylcholine receptors (nAChRs) are found in many central nervous system and nerve-skeletal muscle postsynaptic membranes. The nAChR is a (pseudo) symmetric pentameric structure comprised of homologous subunits. Furthermore, the α subunits exist in at least ten different subtypes (α1 through α10) and the β subunits exist in at least four subtypes (β1 through β4) [1].

In this past year we've initiated an effort to combine several novel techniques to directly optically image the microorganization of AChRs expressed in *Xenopus* oocytes. By expressing different color XFPs in the AChR α and β subunits, we expect to directly resolve and thereby determine the number of α and β subunits that comprise individual receptors. Furthermore we hope to discretely identify the individual receptors over a field-of-view of at least one micron. This will enable us to simultaneously characterize the large-scale distribution of such receptors, to identify any local structures within an imaged membrane surface, and to assay the variation of receptor subtypes.

The techniques being combined in this experiment include a novel method for repeatedly locating imaging probes to specific substrate locations within a few 10's of nm [2], and an ~10 nm resolution, single-molecule sensitive near-field optical microscope developed in collaboration with the Quake group [3,4]. A technique for attachment protein patterning on glass coverslips via Dip-Pen Nanolithographic (DPN) pioneered by the Collier group [5,6] will be used to rigidly adhere the membranes to be image to a glass substrate. Receptor subunit imaging is enabled by the development of specific XFP-labeled AChR subunits [7,8] in the Lester group.

By developing the ability to directly image each subunit of each AChR expressed in the membrane of *Xenopus* oocytes, we lay the groundwork for future studies of transmembrane proteins, receptors and membrane-embedded proteins in many different types of cells. In particular we will substantially advance our ability to image the microorganization of nicotinic acetylcholine receptors at neuronal synapses.

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108. Characterizing the muscle nAChR extracellular M2 conformational transitions

Rigo Pantoja, Mohammed I. Dibas, Dennis A. Dougherty[*]

An environmentally-sensitive fluorescent group was tethered to cysteine mutants near the extracellular end of the mouse muscle nicotinic acetylcholine receptor (nAChR) βM2 and αM2, to monitor conformational changes during the transitions that occur upon agonist binding and lead to pore opening. Two-electrode voltage-clamp recordings revealed that either βM2 L270C (L17'C), βM2 D273C (D20'C), αM2 V259C (V17'C), or αM2 E262C (E20'C) mutations were functionally expressed in *Xenopus* oocytes. A confocal laser scanning microscope (CLSM) with a spectral detector was used to characterize the accessibility of the αV17'C and βM2 L17'C residues to MTS-TAMRA (50 μM) in the absence or presence of ACh (100 μM). The βM2 L17'C side chain was accessible to MTS-TAMRA in the presence, but not in the absence, of ACh. The non-fluorescent thiol reactive N-(2-[(t-Boc)amino]ethyl Maleimide (tBocMal) (50 μM) molecule did not access αV17'C and βM2 L17'C in the absence of ACh: Subsequent labeling by MTS-TAMRA in the presence of ACh (100 μM) was unaffected. Hence, tBocMal was used to mask surface-exposed non-receptor cysteine residues in order to isolate specific labeling by subsequently applied MTS-TAMRA. We conducted simultaneous two-electrode voltage-clamp electrophysiology and fluorometry studies at all four sites. Based on the CLSM results, the oocytes expressing the 17'C mutants were treated with a two-step labeling procedure (1) tBocMal (50 μM) and (2) MTS-TAMRA (50 μM) / ACh (100 μM). The oocytes expressing the 20'C mutants were treated with MTS-TAMRA (50 μM) / ACh (100 μM). MTS-TAMRA attached to any of the four side chains reported an increase in fluorescence (ΔF/F ~ 2 - 3%) upon activation with ACh (10 - 1000 μM). Previously, we showed that β19'C labeled with MTSR also leads to increased fluorescence upon receptor activation. The fluorescence increase of MTS-TAMRA tethered to these positions provides new constraints on the nature of the primary conformational transition at this location.

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109. Detection of a single fluorescent unnatural amino acid incorporated into the nAChR

Rigo Pantoja, Mohammed I. Dibas, James E. Petersson[*]*, Erik A. Rodriguez*[*]*, Dennis A. Dougherty*[1]

Fluorescent unnatural amino acids (fUAAs) represent an attractive alternative for labeling ion channel proteins with minimal structural and amino acid sequence perturbations. Here we report the first successful incorporation of a fUAA, Lys(Bodipy), into the nicotinic acetylcholine receptor (nAChR) using the frameshift suppression methodology. *Xenopus* oocytes were injected with the frameshift suppressor aminoacyl-tRNA ($YFaFS_{ACCC}$-Lys(Bodipy)) and nAChR (α/β19'GGGU/ /) mRNAs. Two-electrode voltage-clamp recordings carried out two days after injection confirmed the presence of functional surface-expressed nAChRs. The measured EC_{50} for the nAChR β19'GGGULys(Bodipy) was 40.16 ± 2.99 μM (n = 5) and is not dramatically different from the known wild-type nAChR EC_{50} (~25 μM). Total internal reflection fluorescence (TIRF) microscopy was used to measure fluorescence from oocytes expressing the nAChR β19'GGGULys(Bodipy). TIRF microscopy was essential for minimizing background fluorescence emanating from oocyte autofluorescence and unincorporated Lys(Bodipy). Time-series photobleaching experiments determined that 70 % of observed sparse puncta (< 0.25 puncta/μm^2) originated from single molecules since a discrete decrease in fluorescence resulted upon photobleaching. The average single-molecule signal to background (ΔF/F) with the corresponding standard deviation (± SD) was approximately 0.38 ± 0.15. Puncta were detected in oocytes injected with only $YFaFS_{ACCC}$-Lys(Bodipy), however, in lower densities (~0.1 puncta/μm^2) and with weaker fluorescence signals (ΔF/F ± SD = 0.20 ± 0.07). To confirm that the puncta originated from Lys(Bodipy) incorporated into a nAChR, we incubated oocytes with alpha-Bungarotoxin mono-conjugated with Alexa488 (α-Btx-Alexa488). The nAChR has two α-Btx-binding sites, thus, if a nAChR β19'GGGULys(Bodipy) is labeled with two α-Btx-Alexa488 probes one would predict observing three discrete steps of photobleaching. Three days after injection, receptors containing the Lys(Bodipy) labeled with two α-Btx-Alexa488 yielded three discrete photobleach steps in 13% of the puncta. In addition, we performed similar experiments with a nAChR (α/β/ / $_{eGFP}$) mutant to confirm the results from the β19'GGGU mutant. Oocytes expressing (α/β/ / $_{eGFP}$) nAChRs at similar levels as they did the β19'GGGULys(Bodipy) nAChRs also produced analogous puncta densities and three discrete photobleach steps from receptors labeled with α-Btx-Alexa488.

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110. *cis-trans* Isomerization at a proline opens the pore of a neurotransmitter–gated ion channel

Sarah C.R. Lummis[1], Lori W. Lee[2], Dennis A. Dougherty[2], Henry A. Lester

The Cys-loop receptor superfamily includes the 5-hydroxytryptamine receptor (5-HT_3), and the nicotinic acetylcholine receptor (nAChR). Neurotransmitter binding in these proteins triggers the opening (gating) of an ion channel, by means of an as yet uncharacterized conformational change. We previously have shown that a specific proline (Pro 8*), located at the apex of the loop between the 2nd and 3rd transmembrane helices (M2-M3), can link binding to gating through a *cis trans* isomerization of the protein backbone. Using unnatural amino acid mutagenesis, a series of proline analogues with varying preference for the *cis* conformer was incorporated at the 8* position of 5-HT_3R. Proline analogues that strongly favor the *trans* conformer produced non-functional channels. Among the functional mutants there was a strong correlation between the intrinsic *cis trans* energy gap of the proline analogue and the activation of the channel, suggesting that *cis trans* isomerization of this single proline provides the switch that interconverts the open and closed states of the channel. We are currently performing the same "prolyl" scan in nAChR. Initial results suggest a potential role for Pro 1*, not Pro 8*, in the gating of nAChR. Our studies are currently aimed at determining if a molecular rearrangement at Pro 1* of nAChR, namely *cis trans* isomerization, is the structural mechanism that opens the receptor pore.

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111. Ligand-receptor interactions in the 5-HT3 receptor

Kiowa S. Bower, Henry A. Lester, Dennis A. Dougherty**

Homology modeling and biochemical studies have been instrumental in advancing our understanding of the 5-HT3 ligand-binding site. Despite the success of these studies, several important aspects of the agonist-receptor binding interaction remain poorly understood. One notable interaction is the putative hydrogen bond between 5-HT3 and the hydroxyl group of the natural agonist serotonin. We aim to identify and characterize novel binding-site interactions with serotonin and other 5-HT3 agonists such as mCPBG. Because receptor gating is thought to begin with a conformational change in the agonist-binding site, a second goal of this study is to characterize residues around the binding site that participate in important interactions during this initial structural rearrangement. Conventional mutations are used as a starting point in our investigations. Residues that appear to play a role in agonist binding or receptor gating will be studied in greater detail using the unnatural amino acid mutagenesis methodology. This methodology will be used to probe the interaction of interest at a more chemical scale than is possible with conventional mutagenesis, and thus provide more convincing evidence. Ligand modification is another tool we use to help us better understand binding interactions. By characterizing the effect of variations to ligand structure, we can gain further insights into specific interactions between agonist and the receptor.

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112. Simultaneous electrophysiological and fluorescence measurements on the glycine receptor M2-M3 linker domain

Stephan A. Pless, Mohammed I. Dibas, Joseph W. Lynch**

How is the binding of a ligand molecule in the N-terminal ligand-binding domain communicated to the channel gate in the transmembrane segments of pentameric Cys-loop receptors? To address this question a previous study has shown that an environmentally-sensitive rhodamine fluorophore attached to the 19' position in the nicotinic acetylcholine receptor (nAChR) α subunit can detect agonist-binding in the ligand-binding domain. Here, we demonstrate specific labeling of a cysteine residue introduced at the 19' position (R271C mutant) of the glycine receptor (GlyR) α1 subunit with a rhodamine fluorophore. Current and fluorescence changes were simultaneously monitored during the application of glycine, strychnine and taurine. This provides crucial insights into the conformational changes accompanying GlyR activation and deactivation. Both the results from glycine and strychnine application indicate a close coupling between movements at the channel gate and the 19' residue in the M2-M3 linker domain. This supports the idea of a concerted movement of a large portion of the M2 helix during glycine receptor activation and deactivation. However, the application of taurine evokes no currents but an increase in fluorescence. Taurine may, thus, induce a conformational change in the M2-M3 linker domain that is distinct from that induced by glycine and strychnine.

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113. Study the gating mechanism in $GABA_C$ receptors

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$GABA_C$ receptors, expressed predominantly in vertebrate retina, are members of the Cys-loop superfamily of ligand-gated ion channels. Upon agonist binding, the receptor undergoes a structural transition from the closed to the open state, but the mechanism of gating is not well studied. Recent work has demonstrated a specific proline (Pro8*), located at the apex of the loop between the M2 and M3 helices, functions as a hinge for movement of M2 during the gating process in 5-HT_{3A} receptor. In contrast, anionic receptors in this superfamily, such as the $GABA_C$ receptor, do not contain this proline, and the possibility of a *cis trans* isomerization at a non-proline receptor should be explored. In this work, we investigate the structural

basis for functionally coupling agonist binding with gating domains in $GABA_C$ receptor by inserting a proline residue in different positions in M2-M3 loop resulting in non-functional receptors. Trying to rescue the receptor by mutation D94N in loop2 failed, except one construct D(11*insert)P/D94N could induce 100nA maximal current. Only S4* in this region can be mutated to proline without greatly effecting channel function, showing a 3-fold increase in EC_{50}. Incorporated two-proline analogues, aze and pip, at this site, produced a 10- and 3-fold increase in EC_{50}, respectively. So we concluded that this mutant proline could not function as the key proline in $5\text{-}HT_{3A}$ as expected. We also generated a chimaeric receptor composed of the gating interface, including loop2, loop7, loop9, PM1 and M2-M3 loop, from the $5\text{-}HT_{3A}$ receptor inserted into the $GABA_C$ receptor. The chimera is expressed and folded correctly in the cell membrane; however, the channels were constitutively active. Future studies will explore the functions of this chimeric receptor.

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114. A novel cation-π interaction in loop A of the $GABA_A$ receptor-binding site

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The binding pockets of Cys-loop receptors are dominated by aromatic amino acids. In the $GABA_A$ receptor α_1Phe65, β_2Tyr97, β_2Tyr157 and β_2Tyr205 are present at the β_2/α_1 interface and likely form an important part of the GABA-binding site. Here we have probed interactions of these residues using subtle chemical changes: Unnatural amino acid mutagenesis was used to introduce a range of Phe analogues, and mutant receptors expressed in oocytes were studied using voltage clamp electrophysiology. Serial mutations at $\beta_2$97 revealed a 20-fold increase in EC_{50} with the addition of each fluorine atom, establishing a cation-π interaction between GABA and this residue. This is the first example of a cation-π interaction in loop A of a Cys-loop receptor, and along with earlier studies that identified cation-π interactions in loop B and loop C, the result emphasizes the adaptability of Cys-loop receptors in response to changes in agonist structure. The data further show that $\alpha_1$65 is relatively tolerant to substitution. Conversely, mutating either β_2Tyr157 or β_2Tyr205 to Phe substantially disrupts receptor function. Substitution of 4-F-Phe, however, at either position, or 4-MeO-Phe at β_2Tyr157 resulted in receptors with wild-type EC_{50}s, suggesting a possible hydrogen bonding interaction. The high precision insights provided by these data allow the construction of an atomic-scale model for GABA docking to the agonist-binding site of the $GABA_A$ receptor.

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115. Probing the Mg^{2+} blockade site of an NMDA receptor with unnatural amino acid mutagenesis

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The NMDA receptor plays a central role in learning and memory in the mammalian CNS. At normal neuronal resting membrane potentials, the pore of this glutamate-gated ion channel is blocked by a Mg^{2+} ion. Previous work suggests that the Mg^{2+}-binding event is novel, involving several asparagine residues and a cation-π interaction between Mg^{2+} and a conserved tryptophan in the pore. Using unnatural amino acid mutagenesis we show that no such cation-π interaction exists. The implicated tryptophan instead appears to play a structural role that can only be fulfilled by a rigid, flat hydrophobic residue. This is the first demonstration of unnatural amino acid incorporation in the NMDA receptor, and it opens the way for future investigations of this pivotal neuroreceptor.

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116. A cation-π interaction between extracellular TEA and an aromatic residue in potassium channels

Christopher A. Ahern[1], Amy L. Eastwood[2], Dennis A. Dougherty[2], Richard Horn[1]

Voltage-gated potassium channels are responsible for returning an excitable cell membrane to a hyperpolarized resting potential after an action potential. High-affinity blockade by extracellular TEA requires the presence of an aromatic residue, either tyrosine or phenylalanine, at position 449 (in Shaker) which sits at the external entrance of the permeation pathway. We investigated whether a cation-π interaction between TEA and Phe449 contributes to TEA block, using the *in vivo* nonsense suppression method to incorporate a series of increasingly fluorinated Phe analogues at position 449. Consistent with a cation-π interaction, increasing fluorination monotonically increased the inhibitory constant Ki for TEA block, the increase reaching 153-fold for 3,4,5F-Phe. An even larger increase in Ki was observed when the benzene ring of Phe449 was substituted by non-aromatic cyclohexane. These results support a strong cation-π component to the TEA block. However, the homologous aromatic residue Tyr82 in crystal structures of KcsA has an orientation incompatible with a cation-π mechanism. We propose that the aromatic residue at this position in voltage-gated potassium channels is more favorably oriented for a cation-π interaction along the permeation pathway. This proposal is supported by high-level *ab initio* calculations of the effects of Phe modifications on TEA-binding energy.

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117. Investigating cation-π interactions in the local anesthetic block of voltage gated sodium channels

Christopher A. Ahern[1], Amy Eastwood[2], Dennis A. Dougherty[2], Richard Horn[1]

Voltage gated sodium channels control the upstroke of the cardiac action potential and are therefore, important for cardiac electrical excitability. Class I anti-arrhythmic drugs, like lidocaine, block sodium conduction in this family of ion channels and are commonly used to treat numerous life threatening conditions, including ventricular fibrillation. We investigated the possibility of a cation-π interaction between lidocaine and the voltage gated sodium channel $rNa_V1.4$. To this end, a series of phenylalanine derivatives deactivated in the cation-π interaction by the addition of fluorine substituents were inserted at aromatic amino acid sites in a transmembrane segment (S6 of domain 4) that lines the internal pore of the channel, using the *in vivo* nonsense suppression methodology. The effect of 200 μM lidocaine was investigated using two-electrode voltage clamp recordings from *Xenopus* oocytes expressing sodium channels carrying these unnatural amino acid derivatives. The nature of the channel block was investigated for steady state tonic conditions and with a use-dependent protocol employing repetitive 20-ms depolarizations from -100 mV to 0 mV over a range from 2 to 20 Hz. For one of these sites, Phe1579, the lidocaine block was highly sensitive to aromatic fluorination. The use-dependent block was successively alleviated by addition of multiple fluorine substituents. Furthermore, suppression at this site with the unnatural amino acid cyclohexylalanine, a side chain sterically similar to phenylalanine but lacking aromatic character, abolished use-dependent block by lidocaine at frequencies as high as 20 Hz. WT channels are roughly 90% blocked under similar conditions. Two other aromatic sites in this S6 segment, Tyr1574 and Tyr1586, showed no effect or mild relief of block, respectively, with the same series of unnatural amino acids. Our results suggest a cation-π contribution to lidocaine block of voltage gated sodium channels.

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118. Nicotine responses in Leu9'Ala and α4 KO mice predict tolerance and sensitization

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Behavioral tolerance and sensitization are two hallmarks of nicotine dependence that underscore long lasting physiological changes associated with chronic nicotine exposure. Alpha 4-containing nicotinic acetylcholine receptors (α4* nAChRs) represent the most widely expressed high affinity nicotinic receptor in the CNS although the precise role of α4* nAChRs in these phenomenon are unclear. To elucidate the contribution of α4* nAChRs in the development of tolerance and sensitization, wild-type, Leu9'Ala α4, or α4 knockout mice were injected with nicotine and their behaviors compared. Repeated selective activation of α4* nAChRs in Leu9'Ala mice produced profound tolerance to hypothermia, whereas no tolerance was observed in α4 KO animals. The summed response from these two mouse lines was not significantly different from the wild-type tolerance profile. In addition, sensitization to nicotine's locomotor effects were observed with activation of α4* nAChRs, while only nicotine-induced hypolocomotor effects were seen in α4 KO mice. The averaged response between the two lines reflected the locomotor response in wild-type animals. Finally, quantitation of radiolabeled cytisine sensitive nAChR binding in Leu9'Ala animals after nicotine treatments showed no significant differences in expression levels compared to saline treated mice even though significant tolerance and sensitization occurred. These data indicate that, by comparing nicotinic responses in two complementary mouse lines, one lacking α4* nAChRs, the other expressing hypersensitive α4* nAChRs, we could accurately predict wild-type tolerance and sensitization. Together our data suggest that activation of α4* nAChRs is both necessary and sufficient for the expression of tolerance and sensitization, and that these behaviors occur in the absence of nAChR upregulation.

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119. Possible D2R-α4* nAChR interactions in a novel mouse model of Parkinson's disease

Andrew R. Tapper, Herwig Just, Cheng Xiao

Strong epidemiological data indicates Parkinson's disease (PD) is less prevalent in smokers. In addition, animal studies have found that nicotine, the addictive component of tobacco smoke, protects DAergic neurons from chemical insult. Thus, nicotine has emerged as a potential neuroprotective agent in PD. Nicotinic receptor modulation of the meso-striatal dopaminergic pathway is complex. Nicotine has been shown in midbrain slices to increase DA release in striatum by activating midbrain DAergic neurons directly via nAChRs. In addition, glutamatergic and GABAergic afferents also express nAChRs and provide excitatory and inhibitory input, respectively, into substantia nigra pars compacta. More recent studies indicate that α4β2* nAChRs may functionally interact directly with D_2 autoreceptors, G-protein coupled DA receptors that are expressed in DAergic neurons and negatively regulate activity. To explore this possible interaction, we injected mice expressing hypersensitive α4* nAChRs (the Leu9'Ala line) with the D_2 receptor agonist, quinpirole, and compared resulting behaviors to wild-type animals. One hour post injection of 5 mg/kg quinpirole, Leu9'Ala mice are significantly hypoactive in a novel environment compared to wild-type animals. In addition, rigidity of fore- and hind-limbs sets in. The severity and duration of this phenotype is quantified in the rotorod assay. When placed on a rotorod rotating at only 3 RPM, Leu9'ala mice

injected with quinpirole fall from the apparatus quickly (latency to fall = ~35 sec. compared to wild-type 180 sec cut off = 180 sec) due to their rigidity, whereas wild-type mice have little trouble staying on the apparatus. Leu9'Ala mice develop a resting tremor that becomes constant one hour post quinpirole injection. In addition these mice also exhibit ptosis. All of these symptoms are reversible; 3-4 hours post injection Leu9'Ala mice are indistinguishable from wild type. This phenotype is rescued by administration of amphetamine and D1 agonists. Together, these data indicate that the Leu9'Ala Parkinsonian phenotype (bradykinesia, rigidity, and resting tremor) is caused by DA depletion. Furthermore, we hypothesize that D_2 activation in our hyper sensitive α4* nAChR mouse model uncovers a novel interaction between Gi/o coupled receptors and α4* nAChRs. Upcoming experiments utilizing neurochemistry and slice electrophysiology will test this hypothesis. It is anticipated that the results from these experiments will not only provide a new pharmacological, reversible, Parkinson's disease mouse model, but also increase understanding of nicotinic receptor mediated modulation of DAergic neurotransmission.

120. Nicotine-induced changes in α4 nicotinic acetylcholine receptor subunit levels in knock-in mice

Raad Nashmi, Purnima Deshpande, Cesar Labarca, Sheri L. McKinney, Sharon Grady, Qi Huang, Tristan McClure Begley*, Allan C. Collins, Michael J. Marks**

α4-containing (α4*) nicotinic acetylcholine receptors (nAChRs) are abundant in the brain. We wish a) to monitor the subcellular and neuroanatomical localizations of α4 nAChR subunits when they are correctly expressed in living cells, and b) to observe their changes in expression with chronic nicotine exposure. Using homologous recombination, we engineered knock-in mice that express a4 nAChR subunits containing yellow fluorescent protein in the M3-M4 cytoplasmic loop (α4YFP). Midbrain neurons cultured from aYFP mice displayed WT levels of dihydro-β-erythroidine-sensitive nicotine-induced currents and ACh-induced Ca^{2+} influx. Synaptosomes from αYFP mice displayed WT levels of striatal cytisine-sensitive and –insensitive ^{125}[I]epibatidine binding, cortical ACh-induced Rb^{+} influx, hippocampal ACh-induced GABA release, and striatal ACh-induced dopamine release. Confocal microscopic imaging and spectral unmixing allows us to isolate α4YFP fluorescence in various brain regions. The strongest α4YFP fluorescence was detected in neurons from the medial habenula. α4YFP could be traced from axonal fibers in the fasciculus retroflexus emanating from the medial habenula and terminating in the interpeduncular nucleus. Strong α4YFP expression was found in soma and neurites throughout the thalamus, in axonal fibers in the internal capsule, and also in fibers coursing between dopaminergic fibers of the caudate-putamen. α4YFP fluorescence was ~2-fold more intense in the cell bodies and dendrites of dopaminergic neurons (substantia nigra pars compacta, ventral tegmental area) than in neighboring GABAergic neurons. Mice were implanted subcutaneously with mini-osmotic pumps that delivered either saline or nicotine (2 mg/kg/hr for 10 d). The nicotine-treated mice showed alterations in α4YFP fluorescence in specific brain regions. These α4YFP mice provide an opportunity to quantify α4* nAChRs at μm resolution in the CNS and to study their role in nicotine addiction.

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121. Spontaneous seizures and nicotine-induced c-fos in knock-in mice with hypersensitive α4* nicotinic receptors

Carlos Fonck

Autosomal dominant frontal lobe epilepsy (ADNFLE) is a rare epileptic syndrome characterized by brief, sometimes violent partial seizures, occurring in sleep and starting during childhood. ADNFLE is linked to point mutations in the neuronal nicotinic acetylcholine receptor (nAChR) α4 and β2 subunits. We previously showed that mice expressing gain-of-function α4-containing (α4*) nAChRs (Leu9'Ala, not an ADNFLE mutation), following nicotine (nic) injections, displayed a syndrome with partial seizures similar to those experienced spontaneously by α4-linked ADNFLE patients (see reference). We now report the occurrence of spontaneous seizures in several Leu9'Ala mice 12 to 29 days old. Spontaneous seizures in 11 homozygous and one heterozygous Leu9'Ala mice to date were characterized by loss of righting response, repetitive jerky movements of forelimbs and hindlimbs and flailing or stiff tail. Seizures were followed by rigidity of the body and extremities, disorientation, lack of locomotion and hypothermia. Convulsive bouts typically lasted between 10 to 20 min. Three of the mice died within an hour after seizure onset, while the rest of the animals recovered completely. In order to identify neuronal populations activated during seizures, we are measuring the levels of c-fos expression in the brains of wild-type (WT) and Leu9'Ala mice that undergo seizures following nic treatment, as well as mice that experience spontaneous seizures. Seizures triggered by 10 mg/k nic in WT and Leu9'Ala result in strong c-fos expression in the cerebral cortex, the pyriform cortex, the dentate gyrus and the media habenula. At 2 mg/kg nic, WT mice experience no seizures and display little c-fos staining, whereas Leu9'Ala mice have partial clonic seizures and show strong c-fos label in the medial habenula. Spontaneous seizures in Leu9'Ala mice, thus far, cause increases in c-fos staining in the cerebral cortex and the laterodorsal thalamic nucleus, but little or no expression in the medial habenula.

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122. Region-specific functional expression of α4-containing nicotinic receptors in the medial habenula

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The medial habenula (MHb) is the brain nucleus with the highest expression level of neuronal nicotinic acetylcholine receptors (nAChRs). Although mRNAs for several nAChR subtypes have been detected in the MHb, it is not yet known which of these subunits form functional receptors. We investigated the expression pattern and function of α4-containing (α4*) nAChRs in the MHb with three complementary experiments: a) Knock-in mice that express fluorescent α4-containing nAChRs show fluorescence in several brain regions (α4YFP, Nashmi *et al*., SFN, 2005 and 2006), but the most intensely YFP-labeled neurons were concentrated in the ventral-lateral portion of the MHb (VL-MHb); b) In knock-in mice that express mutated hypersensitive α4* nAChRs (Leu9'Ala), injections of either 0.2 mg/k or 2 mg/k nicotine led to strong c-fos expression in VL-MHb. No such pattern of c-fos expression was observed in the MHb of wild-type mice. Therefore, at relatively low nicotine doses, neurons expressing hypersensitive α4* nAChRs are activated; and, c) Patch-clamp experiments showed that VL-MHb neurons from Leu9'Ala mice responded robustly (166 ± 37 pA) to pressure applied 1 μM nicotine, whereas neurons in the medial portion of the MHb had little or no responses (< 15 pA). These nicotine responses in Leu9'AlaVL-MHb were ~10-fold higher than in WT VL-MHb. Our results indicate that functional α4* receptors are expressed within the MHb, and that this expression is specific to the VL subregion of MHb.

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123. Increased sensitivity to nicotine-induced seizures in beta2 V287L knock-in mice

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Autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) is partial epilepsy characterized by brief motor seizures that predominantly occur during sleep. Several mutations in two genes coding for the nicotinic acetylcholine receptor (CHRNA4 and CHRNB2) have been found in ADNFLE patients. One of these is a missense mutation of valine 287 to leucine in the M2 region of the beta2 subunit. Using the gene targeting technique, we generated "knock-in" mice carrying the same V287L mutation found in human ADNFLE. Beta2 V287L mice are born at an expected Mendelian ratio and appear normal. There were no apparent neuroanatomical differences between V287L and wild-type mice in the brain regions examined. Spontaneous seizures were not frequently observed in beta2 V287L mice. However, V287L mice showed several-fold higher sensitivity to seizures induced by nicotine. V287L mice also displayed hypersensitivity to nicotine-induced dorsiflexion of the tail (Straub tail). Thus, beta2 V287L mice may be a useful animal model for understanding the pathophysiology of ADNFLE.

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124. Balancing neuronal activity and survival via the cholinergic modulator lynx1

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Nicotinic acetylcholine receptors (nAChRs) affect a wide array of biological processes including learning and memory, attention, and addiction. Lynx1, the founding member of a family of mammalian prototoxins, modulates nAChR function *in vitro* by altering agonist sensitivity and desensitization kinetics. Here we demonstrate, through the generation of lynx1 knockout (lynx1 KO) mice, that lynx1 modulates nAChR signaling *in vivo*. Its removal causes hypersensitive nAChRs, decreasing the EC50 for nicotine by ~10-fold. Decreased desensitization is shown by elected intracellular calcium levels in neurons in response to nicotine. Lynx1 KO mice also showed decreased nicotine-induced paired pulse facilitation. In addition, lynx1 KO exhibit enhancements in performance in tasks measuring learning and memory. Consistent with reports that mutations resulting in hyper-activation of nAChRs can lead to neurodegeneration, aging lynx1 null mutant mice exhibit a vacuolating degeneration that is exacerbated by nicotine and ameliorated by null-mutations in nAChRs. We conclude that lynx1 functions as an allosteric

modulator of nAChR function *in vivo*, and that it plays a critical role in balancing neuronal function and survival in the CNS.

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125. Interactions between the prototoxin lynx1 and $\alpha_4\beta_2$ nicotinic acetylcholine receptors

Cagdas D. Son

The mammalian prototoxin lynx1 shares an evolutionary relationship with the Ly-6/neurotoxin gene family. Similar to α-neurotoxins, such as the bungarotoxins and muscarinic toxins, lynx1 predicted to adopt the three-fingered toxin fold. lynx1 is expressed in large projection neurons in the hippocampus, cortex, and cerebellum [1]. lynx1 co-localizes with nicotinic acetylcholine receptors (nAChRs) on CNS neurons and physically interacts with these receptors [2]. Studies with $\alpha_4\beta_2$ nAChRs revealed that lynx1 modulates current amplitudes, enhances desensitization and increases the EC_{50} for activation by Ach [3]. These results suggest that lynx1 opposes the changes induced by chronic exposure to nicotine. Recent work by Sekhon *et al*., 2005, showed that lynx1 expression in lungs increased following nicotine exposure [4]. Thus, we hypothesize that interactions between lynx1 and nAChRs are important in the response to chronic nicotine exposure *in vivo*.

In order to identify the important residue(s) and/or region(s) in lynx1 function we will examine Ach-induced macroscopic current responses of $\alpha_4\beta_2$ nAChRs using two-electrode voltage clamp in the presence of wild-type or mutant lynx1. Alternatively, mutants of a_4b_2 will be generated and expressed in *Xenopus* oocytes and tested for wild-type lynx1 binding by competition-binding assays and surface cysteine accessibility method to locate the residues important in lynx1 interaction on the receptor. In addition, direct interaction(s) between neuronal nAChRs and lynx1 will be examined by generating a covalent bond between these two proteins. Using the unnatural amino acid replacement techniques we will introduce a photoaffinity crosslinker (such as p-benzoylphenylalanine) in the regions of receptor or lynx1 that are predicted to be involved in the interaction and induce crosslinking by light activation. The resultant complex will then be analyzed by fragmentation and protein sequencing by mass spectrometry to identify the crosslinked residue on the partner. These studies will help us better understand the interactions between lynx1 and neuronal nAChRs, revealing important insights regarding how lynx1 modulates the functional properties of nAChRs.

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126. *In vivo* reversible silencing of striatal neurons by an ivermectin gated Cl channel

Walter Lerchner, Eric M. Slimko, Cheng Xiao, Laurent van Trigt, David J. Anderson[*]

To develop tools for molecularly dissecting defined neuronal circuits in the brain we have tested a two component ion channel-based silencing system: expression of an ivermectin (IVM)-gated glutamate-sensitive Cl channel from *C. elegans.* The heteropentameric channel requires both alpha and beta subunits, allowing for combinatorial strategies to increase the specificity of expression. Both subunits have been codon optimized, rendered insensitive to glutamate and tagged with CFP (alpha) and YFP (beta). To test the system in the CNS *in vivo*, we injected mice unilaterally in the striatum with a suspension of two recombinant viruses (AAV2) expressing the alpha and beta subunits. IVM-induced silencing was confirmed in a simple rotation assay. Ten days after virus injection, the mice were injected intraperitoneally with IVM and tested at various time points for amphetamine-induced rotation. If dopaminergic transmission via the striatum is unilaterally disrupted in amphetamine-injected animals, ipsilateral rotation occurs. Such rotation was observed in animals coinjected with virus encoding the alpha and beta subunit, but not with virus encoding the alpha or beta subunit alone. With injections of 10 mg/kg IVM, the rotation appears after 4 h, peaks at 12 h and remains amphetamine-inducible for up to 3 d. We repeated IVM administration and withdrawal at least twice in the same animals. While repeated IVM administration restores the full rotation phenotype, in several mice some amphetamine-inducible rotation remains after IVM withdrawal. Further experiments will determine whether this effect is caused by behavioral sensitization, or by a percentage of neurons that cannot be re-activated after prolonged silencing. Striatal slice patch recordings show that ~50% of neurons from virus-injected striatum, but none from uninjected striatum, respond to 10-20 min 10 nM IVM with hyperpolarization and reduced firing, as expected from Cl channel activation.

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127. α6* nicotinic ACh receptors: Role in nicotine addiction

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Addiction to cigarettes is a major health risk to smokers and non-smokers alike, and has created a massive healthcare burden for developed countries. We hypothesize that interfering with the establishment and/or maintenance of nicotine dependence, specifically within the midbrain dopaminergic system, will help to foster smoking cessation. Several nicotinic ACh receptor

subunits are expressed in this brain region, including α4, α6, β2, and β3. α6-containing (denoted as α6*) nicotinic ACh receptors are required for 50-70% of total nicotine-stimulated dopamine release, an important neurochemical aspect of dependence to nicotine. To further understand the role of α6* nAChRs in nicotine addiction, we will amplify and isolate α6-dependent behavioral and cellular responses in mice by creating a new mouse strain expressing hypersensitive α6 subunits. α6 subunits are expressed poorly outside of their native neurons in the central nervous system, which precludes a detailed *in vitro* study aimed at designing a suitable hypersensitive mutant α6* receptor. To circumvent this problem, we have begun to create the desired mouse strain using BAC (bacterial artificial chromosome) transgenesis. In exon 5 of the α6 gene, the 9' position of TM2 will be mutated from Leu to Ser or Ala, and the mutant BAC construct will be incorporated into the mouse genome via random insertion following pronuclear injection. Founders will be screened for transgene expression. Using nicotine-stimulated dopamine release from synaptosomes, along with fura-2 Ca^{2+} imaging and patch-clamp electrophysiology from primary neurons, we will determine the extent of hypersensitivity across several L9'S and L9'A lines of low and high transgene copy number. Promising lines will be assayed for hypersensitivity to nicotine-dependent reward, tolerance, and withdrawal in behavioral assays. With the ability to selectively activate α6* receptors, these mice will allow us to determine which aspects of nicotine addiction α6* receptors are sufficient for. In addition, these mice will be useful for smoking cessation lead compound selection and optimization studies that focus on α6* receptors.

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128. hERG block in acquired long-QT Syndrome probed with unnatural amino acids

Fraser J. Moss

Acquired LQT syndrome arising from block of the human ether-a-go-go related gene (hERG) potassium channel has become the primary safety concern when developing new pharmaceuticals. Lead compounds must not block hERG if they are to move forward. Substituting unnatural amino acids (UAAs) for critical hERG residues using nonsense suppression in mammalian cell lines will resolve the nature of the non-covalent interactions between each residue and each drug. For example, di-, or tetra-fluoro derivatives of phenylalanine minimally perturb the Phe structure, but would form a uniform graded series of increasingly electron-withdrawing groups when incorporated at position 656 (wt residue, Phe) or position 652 (wt residue, Tyr) that could probe any cation-π or π-π drug-channel interactions. Traditionally, to incorporate UAAs, we took a stoichiometric approach, where after each tRNA molecule had delivered one UAA molecule it became redundant. We have demonstrated recovery of hERG current with this approach. However, expression levels were less than 10% of wt hERG and often phenotypes were mixed, indicating re-acylation of the tRNA suppressors by endogenous tRNA synthetases after delivering their initial cargo. Consequently, we are developing a catalytic approach; expanding the genetic code of CHO cells to express tRNA synthetases that repeatedly re-acylate orthogonal suppressor tRNAs with UAAs supplied in the culture media. We have identified a tyrosine aminoacyl-tRNA synthetase (bTyrRS)/tRNA ($bTyrtRNA_{CUA}$) pair from bacteria that rescue hERG expression when co-transfected with a hERG Y652TAG mutant in CHO cells. Importantly, if either the tRNA synthetase or the tRNA plasmids are omitted from the transfection, no hERG expression is detected. The biophysical properties of currents rescued by the tyrosine-tRNA synthetase/tRNA pair were not significantly different from WT hERG. Work continues to randomly mutate the bTyrRS at five positions involved in binding tyrosine. The generated mutant library will be screened for UAA incorporation. $tRNA_{CUA}$/aaRS pairs identified in the screen will then be tested for UAA incorporation in hERG channels expressed in CHO-K1 cells

129. Fluorescent mGAT1 constructs reveal important motifs for correct transporter trafficking and dimerization

Fraser J. Moss, Princess I. Imoukhuede, Joanna L. Jankowsky, Jia Hu[*]

We studied fusion proteins of the mouse -aminobutyric acid transporter (mGAT1) and the yellow or cyan fluorescent proteins (YFP or CFP) to understand the density, intracellular processing, PDZ interactions, trafficking, and dimerization of mGAT1. We aimed to create a fluorescent fusion that behaved like wild-type (wt) transporter in all respects to create knock-in mice expressing fluorescent transporters. Species that did not recreate the wt phenotype could highlight regions of the protein that modulate assembly and sub-cellular localization. mGAT1-XFP3, -XFP8, -XFP20, -XFP45 were fusions of XFP (X= C or Y) to the mGAT1 C-terminus with an additional 3, 8, 20 or, 45 residues from the hGAT1 C-terminus fused after the fluorophore. mGAT1-XFP* had an alternative C-terminal PDZ-type II interacting region (YKV) compared to the wt. mGAT1-XFPCT, fused XFP between the penultimate and final exon of mGAT1. We measured [^{3}H]-GABA uptake in N2A cells, in assays optimized for linearity; we recorded the fluorescent resonance energy transfer (FRET) efficiencies when YFP and CFP pairs of the same construct design were co-expressed; biotinylation assays determined the proportion of the expressed transporters localized in the plasma membrane versus the cytoplasm. mGAT1-XFP* and -XFP8-28 showed no significant difference to wt in uptake or biotinylation experiments. Co-expression of mGAT1-XFP* and mGAT1-XFP8 pairs gave FRET efficiencies of 21% and 8% respectively, reasonable values for side-by-side 12 transmembrane (TM) domain transporters. mGAT1-XFPCT and mGAT1-XFP45 had poor uptake and resided predominantly in the

cytoplasm; interestingly, FRET efficiency for the poorly trafficked mGAT1-XFPCT's was 33%, but FRET could not be detected for mGAT1-XFP45. This implied bisecting the 17 amino acids immediately after TM12 with XFP inhibits transporter export to the membrane but not assembly. However, two copies of this same region per GAT1 molecule prevented transporter assembly and export from endoplasmic reticulum. We concluded that mGAT1-XFP* is the most suitable construct to build fluorescent mGAT1 mice because it produced the strongest FRET of all the constructs that behave like wt. Nonetheless, other constructs highlighted regions that regulate normal mGAT1 trafficking and assembly.

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130. Measuring the lateral mobility of GAT1 using fluorescence recovery after photobleaching

Princess I. Imoukhuede[*], Henry A. Lester

We seek an understanding of GAT1 trafficking onto the surface of the neuronal membrane. GAT1 is a membrane protein that reuptakes GABA from the synapse, thus it terminates inhibitory signaling events. It is well known that membrane protein exchange occurs via endocytosis/exocytosis; however, little work has been done to determine the role of lateral diffusion in the regulation of protein number at the plasma membrane. To this end, we utilize fluorescence recovery after photobleaching (FRAP) to determine the mechanics of transporter exchange at the cell membrane. Treatment with latrunculin B, which depolymerizes actin filaments, decreased both the mobility and recovery time constant of GAT1. However, treatment with nocodazole, which depolymerizes microtubules, did not affect the mobility of GAT1. Our data suggests that GAT1 is anchored to the membrane via an association with actin filaments.

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131. Combination therapy for the treatment of Alzheimer's disease

Joanna Jankowsky, Pritam Das[1], David Borchelt[2], Henry Lester, Robert Switzer III[3], Todd Golde[1]

Alzheimer's disease is thought to stem from the over-accumulation of a small peptide called Aβ that aggregates into amyloid plaques in parts of the brain that are critical for learning and memory. Because of its hypothesized role in the onset of AD, considerable effort has been devoted to the development of new treatments for Alzheimer's disease based on the prediction that lowering the amount of amyloid-β peptide (Aβ) in the brain will facilitate the removal of amyloid lesions and repair of damaged tissue. Work towards this goal has been divided between two main approaches. The first uses the immune system to combat high levels of Aβ by either actively immunizing patients against Aβ, or by passively transferring antibodies to it. Meanwhile, other investigators have sought to lower Aβ levels by targeting its production. In isolation each strategy faces substantial limitations; however, combination of the two therapies may overcome their individual shortcomings. We are, therefore, testing a combination of chronic Aβ inhibition with acute antibody administration for the treatment of Alzheimer's in a transgenic mouse model for the disease. Our hypothesis is that acute immunotherapy will reverse pre-existing lesions while chroniFc suppression of new Aβ synthesis will prevent their recurrence. This work will focus on a new mouse model for AD in which we have used a genetic switch to mimic the effect of treatment with secretase inhibitors. These animals express an engineered form of APP that can be regulated by doxycycline. Experiments are underway to test whether acute administration of anti-Aβ antibody in combination with chronic doxycycline treatment to prevent further Aβ synthesis can produce long-term amyloid clearance. Concurrent experiments will compare the effectiveness of this approach in older mice with an aging immune system to that attained in young animals. Together, these experiments could provide strong proof-of-principle that combination therapy might be used to successfully reverse prior amyloid accumulation and prevent its recurrence in patients with Alzheimer's disease.

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132. Selective silencing of mammalian neurons using glutamate-gated chloride channels

Herwig Just, Eric Slimko

We are developing a technique to selectively silence mammalian neurons *in vivo*. This will enable us to study the function of specific types of neurons in the context of an intact brain circuitry. We are heterologously expressing a glutamate-gated chloride channel (GluCl) from *C. elegans* in neurons. The GluCl channel is a heteropentamer consisting of two subunits, an alpha and a beta subunit. These subunits have been modified for our purposes: they have been codon-optimized for enhanced mammalian expression, tagged with fluorescent proteins to allow for direct visualization and had the tyrosine at residue 182 of the GluCl beta-subunit mutated to a phenylalanine to eliminate glutamate sensitivity. Activation of GluCl by the agonist ivermectin (IVM) elicits a current that clamps the neurons to the resting potential of chloride, thereby inhibiting action potentials ('silencing'). High expression levels of the GluCl channel are required for efficient silencing. Our initial transgenic approaches generated mice with appropriate expression of the GluCl channel in neurons according to the promoters from which their expression was being driven. However, the expression levels were not sufficiently high for visualization or to elicit silencing in these neurons. Consequently, we have developed an alternative strategy: Adeno-associated virus encoding, the modified GluCl

subunits to drive high-level expression in the ventral-tegmental area (VTA), are being stereotaxically injected into this region of mouse brains. The VTA is part of the mesocorticolimbic reward pathway. Cells in the VTA send ascending fibers to the nucleus accumbens releasing dopamine (DA). Drugs of abuse increase release of DA in the nucleus accumbens. To block this pathway the virus-injected animals are treated with IVM to inhibit neuronal firing and subsequently administered drugs of abuse (cocaine, amphetamine). Using place preference assays, we are studying the effect of inhibiting neuronal function in the VTA on the development of addiction.

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Senior Research Fellow: Ali Khoshnan
Research Fellows: Sylvian Bauer, Benjamin Deverman, Natalia Malkova
Graduate Students: Walter Bugg, Jennifer Montgomery, Stephen Smith, Amber Southwell
Research and Laboratory Staff: Kathleen Hamilton, Jan Ko, Kelly Lin[1], Doreen McDowell, Limin Shi, Simona Tescu[1], Emma Thomas[1], Erin Watkin, Wensi Xu[1]
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Summary: Much of the research in this laboratory involves the study of interactions between the nervous and immune systems. Using knockout (KO) mice and over-expression *in vivo* with viral vectors, we are exploring the role of the neuropoietic cytokine leukemia inhibitor factor (LIF) in Alzheimer's disease and inflammation. This cytokine is further being used to manipulate neural stem cell proliferation and fate in the brain. Also in the context of neuroimmune interactions, we are investigating a mouse model of mental illness based on the known risk factor of maternal influenza infection. Huntington's disease (HD) is another focus, where we are investigating potential therapies using intracellular expression of antibodies, and manipulating NFκB activity. An additional project involves the inhibition of melanoma and glioma cell growth by endothelin receptor blockers.

Cytokines are diffusible, intercellular messengers that were originally studied in the immune system. Our group contributed to the discovery of a new family that we have termed the neuropoietic cytokines, because of their action in both the nervous and hematopoietic/immune systems. We have demonstrated that one of these cytokines, LIF, can coordinate the neuronal, glial and immune reactions to injury. Using both delivery of LIF *in vivo* and examination of the consequences of knocking out the LIF gene in mice, we find that this cytokine has a powerful regulatory effect on the inflammatory cascade. Moreover, LIF can regulate neurogenesis and gliogenesis. We find that LIF is a critical regulator of astrocyte and microglial activation following stroke, seizure or trauma, and that this cytokine also regulates inflammatory cell infiltration, neuronal and oligodendrocyte death, gene expression, as well as the production of adult neural stem cell renewal. These results highlight LIF as an important therapeutic target. We are also examining the role of LIF in a transgenic mouse model of Alzheimer's disease, where its administration can decrease the level of senile plaques.

Cytokine involvement in a new model for mental illness is also being investigated. This mouse model is based on findings that maternal infection can increase the likelihood of schizophrenia or autism in the offspring. We are using behavioral, neuropathological, molecular and brain imaging methods to investigate the effects of maternal influenza infection on fetal brain development and how this leads to altered behavior in young and adult offspring.

We are utilizing intracellular antibody expression to block the toxicity of mutant huntingtin (Htt), the protein that causes HD. We have produced single-chain antibodies (scFvs) that bind to various domains of Htt, and these can either exacerbate or alleviate Htt toxicity in cultured cells, acute brain slices, and in a *Drosophila* HD model. Work has begun on viral vectors for delivering scFvs in a mouse model of HD. We have also implicated the NFκB signaling pathway in the pathogenesis of HD, and identified several steps in this signaling cascade as potential therapeutic targets.

133. Intrabodies as potential therapeutics for Huntington's disease

Amber L. Southwell, Jan Ko, Ali Khoshnan

Huntington's disease (HD) is a neurodegenerative disorder caused by the expansion of a polyglutamine tract in the huntingtin protein (Htt). This expansion leads to Htt aggregation and degeneration of medium-sized spiny neurons of the striatum. We are investigating the use of intrabodies (intracellularly expressed antibodies) as potential therapeutics for HD. Intrabodies have great therapeutic potential due to their specificity in target recognition, and are currently being tested in the treatment of HIV and cancer. Single chains (scFvs) consisting of the variable heavy and light antigen recognition domains (V_H and V_L) connected by a linker are preferable for this type of application due to their small size and lack of antigenicity. We have developed a number of monoclonal antibody (mAb)-derived scFvs that recognize different epitopes in exon 1 of Htt. The mAb MW7, which recognizes the polyproline stretches in Htt, decreases mutant Htt-induced aggregation and cell death. This effect has been demonstrated in cell culture, acute brain slices (with P. Reinhart and D. Lo at Duke), and in a *Drosophila* model of HD (with G. Jackson at UCLA). This intrabody, however, requires a 4:1 ratio to mutant Htt for its effects. In an effort to generate a more efficacious intrabody with the benefits of binding in the polyproline region, we produced novel intrabodies (Happs) against the proline-rich domain of Htt. The Happs were isolated from a human synthetic phage display intrabody library first, by selection with a peptide from the unique proline-rich sequence located between the two polyproline stretches

and second, with mutant Htt. Happ1 and three are single domain, light chain intrabodies (V_Ls) that bind to mutant Htt in a proline-rich domain-dependent manner. They reduce mutant Htt-induced aggregation and toxicity in cell culture models. They have an optimum 2:1 ratio to mutant Htt but are still effective at 1:1. We then compared Happ1 and three to the single domain, light-chain intrabody V_L12.3, which recognizes the first 17 amino acids of Htt (Colby *et. al.* (2004) *PNAS* **101**:51). V_L12.3 was engineered to fold without disulfide bonds and to reduce mutant Htt-induced aggregation at a 0.5:1 ratio to Htt. Cell culture studies indicate that the beneficial effects of V_L12.3 are mechanistically different from the Happs. While all three intrabodies decrease toxicity and aggregated Htt, unlike V_L12.3, the Happs appear to do so without increasing soluble mutant Htt, suggesting a change in Htt turnover. The therapeutic potential of these intrabodies will next be tested in a mouse model of HD using viral-mediated gene therapy. Insights acquired through such experiments may contribute to the generation of novel therapeutics for HD.

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134. Direct selection of neuroprotective intrabodies for Huntington's disease

Charles W. Bugg, Ali Khoshnan, Paul H. Patterson

Death of neurons in the striatum and cortex is a key component of Huntington's disease (HD), a progressive and fatal neurodegenerative disorder. Several intracellular antibodies (intrabodies) have been developed as potential therapeutics for HD, based on their binding to mutant huntingtin (mHtt) protein. These intrabodies ameliorate the disease phenotype with various efficiencies in cell culture. Some even exacerbate the cell death. We are developing a more direct, iterative system to select for intrabodies that prevent mHtt-induced cell death without necessarily binding mHtt. A library of intrabodies is expressed in PC12 cells carrying mHtt exon 1 (HDx1) with an expanded polyglutamine repeat (103Q) under control of the ecdysone promoter. After induction of the toxic HDx1, only cells carrying neuroprotective intrabodies will survive, and those intrabody sequences can be recovered, amplified and recycled through the selection. The efficiency of the selection will be determined in large part by the ability to kill nearly all cells that do not carry a neuroprotective sequence. After optimization of conditions, we find that 99% of the PC12/PQ103 cells die within 12 days of induction with 5μM Muristerone A. Two library strategies are being pursued. A mutagenized intrabody pool will be used to directly transfect the PQ103-inducible PC12 cells. In parallel, the intrabody library will be cloned into the lentiviral vector pFUGW and used to produce a lentiviral library with which the PC12 cells will be transduced. As proof of principle, we will select a published intrabody that functions in the absence of the conserved disulfide bond. This system will then be used to select novel intrabodies that protect from mHtt-induced cell death for use as therapeutics for HD.

135. Interaction of mutant huntingtin with the NF-κB pathway

Ali Khoshnan, Simona Tescu[1]*, Jan Ko*

Transcriptional dysregulation by mutant huntingtin (Htt) protein has been implicated in the pathogenesis of Huntington's disease (HD) and we found that mutant Htt activates the NF-κB pathway. Mutant Htt physically associates with IKKγ, a regulatory component of the IκB kinase complex (IKK). In cultured cells, this interaction results in the activation of IKK, leading to the phosphorylation and degradation of the inhibitory protein IκBα. These findings have *in vivo* relevance, as striatal extracts from HD transgenic mice have higher levels of IKK than extracts from control mice, and activated NF-κB is found in the nucleus of striatal and cortical neurons in HD mice. Binding to IKKγ is mediated by the expanded polyglutamine stretch in mutant Htt, and is augmented by the proline-rich motifs of Htt. Expression of IKKγ promotes mutant Htt aggregation and nuclear localization. Conversely, an N-terminally truncated form of IKKγ, which interferes with IKK activity, blocks Htt-induced NF-κB activation and reduces the toxicity of mutant Htt in cell culture and in an acute brain slice model of HD. Toxicity is also inhibited by expression of a mutant F-box deleted E-3 ubiquitin ligase, ΔF-βTRCP, which specifically blocks degradation of IκB inhibitory proteins. Thus, aberrant interaction of mutant Htt with IKKγ, and subsequent NF-κB activation, may be important for HD pathology. On the other hand, IKKγ can influence Htt actions through non-NF-κB pathways. Expression of IKKγ promotes the toxicity of full-length mutant Htt, implicating IKKγ as a modifier of mutant Htt toxicity. IKKγ also promotes aggregation/oligomerization of mutant Htt. In neurons, IKKγ is predominantly located in the nucleus. Microarray analysis shows that expression of IKKγ from a recombinant lentivirus influences expression of many genes implicated in neuronal survival and differentiation. Studies are in progress to examine specific genes affected by IKKγ that may regulate the toxicity of mutant Htt and neurodegeneration.

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136. The role of IκB-kinase complex in neuronal development and function

Ali Khoshnan, Jan Ko

The role of IκB-kinase complex (IKK) in neuronal development, survival and degeneration is not understood. In non-neuronal cells IKK regulates the activity of the transcription factor NF-κB. The core components of the IKK complex include two serine-threonine kinases IKKα (IKK1) and IKKβ (IKK2), and a

regulatory non-catalytic module, IKKγ (NEMO). IKKα and IKKγ also have NF-κB independent functions. We showed that binding of mutant Htt to IKKγ leads to aberrant IKK activity and enhances mutant Htt neurotoxicity (Khoshnan *et al*., 2004). On the other hand, mice deleted for both IKKα and IKKβ die at E12 and display enhanced apoptosis in the neuroepithelium, indicating normal and regulated activity of IKK complex is essential for development and function of neurons. We are specifically interested in the function of IKKα, as it has been implicated in chromatin modification in epithelial cells. We find that IKKα promotes neurite outgrowth in differentiating rat cortical stem cells and it is found in the growing tips of neurites. Growth cone cues such as netrin activate IKK. IKKα is activated in response to neuroprotective molecules such as IGF and estrogen, suggesting a role in neuronal survival. IKKα is translocated to the nucleus upon IGF treatment, where it colocalizes with CREB-binding protein. We are studying the mechanism of IKKα regulation of neurite outgrowth, as well as the signaling pathways influenced by the nuclear function of IKKα in neurons and neuroblasts.

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137. Production of anti-amyloid-beta antibodies that bind to various forms of amyloid proteins

Anna S. Gardberg[1], Lezlee T. Dice[1], Elizabeth Helmbrecht[1], Jan Ko, Susan Ou, Paul H. Patterson, David Myszka[1], Ronald Wetzel[1], Chris Dealwis[1]

Alzheimer's disease (AD), one of the most common dementia disorders, has profound medical and social consequences. Synaptic dysfunction, neurofribrillary tangles, neurodegeneration and memory impairment have been found in patients with AD. Amyloid-beta (Aβ), either in soluble or insoluble form, has been hypothesized to be responsible for AD pathology. Both active and passive immunotherapy targeting Aβ have been shown to ameliorate histological and behavioral pathology in AD transgenic mice. Although clinical trials in human with active immunotherapy have been terminated because of occasional cases of meningoencephalitis, a safer vaccine is being sought because of the impressive mouse results. We have generated a panel of monoclonal antibodies to the protofibril or fibril forms of Aβ. The crystallographic structures of two of the antigen antibody-binding pairs have been determined, providing insight into rational design for AD immunotherapy.

[1]*University of Tennessee*

138. Maternal influenza infection alters fetal brain development

Limin Shi, Natalia Malkova, [1]Yixuan Andy Shi, Paul H. Patterson

Epidemiological studies have shown that maternal infection can increase the risk for mental illness in the offspring. In a mouse model of maternal respiratory infection with influenza virus, the adult offspring display striking behavioral, pharmacological and histological abnormalities. In addition to a localized loss of Purkinje cells that is strikingly similar to a common finding in autism, we find an altered migration of neurons in the fetal cortex. Whereas late-born cortical neurons are appropriately positioned in the upper layers in sham control offspring and naïve mice from postnatal day 0 to young adulthood, the late-born cortical neurons in offspring of infected mothers are randomly distributed across all layers. This neuropathology is of particular interest because it is very similar to that reported for mice whose level of Disrupted-In-Schizophrenia-1 (DISC1) mRNA is knocked down. It is possible that our finding in the mouse model is relevant to the pathology and behavioral abnormalities that have been linked to the cerebral cortex in schizophrenia.

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139. The maternal inflammatory response and its effects on fetal development and behavior of the adult offspring

Benjamin E. Deverman, Wendy Xu[1], Sarah Wadsworth[2], Paul H. Patterson

Significant epidemiological evidence indicates that there is an increased incidence of schizophrenia and autism in the offspring of women exposed to viral infection during pregnancy. In a mouse model based on these findings, the adult offspring of mothers exposed to influenza during pregnancy exhibit several behavioral and neuropathological abnormalities that are consistent with those observed in schizophrenia and autism. Moreover, several of these abnormalities is also seen in the adult offspring of mothers injected with poly(I:C), a dsRNA that elicits an inflammatory response similar to that induced by influenza infection.

The inflammatory response to viral infection and poly(I:C) administration is mediated by numerous cytokines, many of which could have profound effects on fetal brain development. Therefore, in an effort to determine which cytokines may be responsible for the observed behavioral and neuropathological abnormalities, we are measuring cytokine induction during the inflammatory response to poly(I:C) in both maternal and fetal tissues. As expected, we have found that the levels of numerous cytokines are strongly induced in the maternal serum during the response to poly(I:C). In addition, we observe a significant increase in several of these cytokines in the local fetal-maternal environment (i.e., the placenta, decidua, yolk sac, and amniotic fluid) during the response to poly(I:C). In contrast, we have not yet detected significantly increased levels of any cytokines examined in

the fetus following poly(I:C) administration. We are currently attempting to increase the sensitivity of our cytokine analysis protocols, assessing maternal and fetal tissues for changes in additional cytokines, and looking for evidence of downstream cytokine signaling pathway activation during the response to poly(I:C) in both the fetus and the fetal-maternal environment. Studies are ongoing to determine the consequences that these alterations in the fetal-maternal environment may have for fetal brain development.

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[2]*Caltech undergraduate student*

140. Interleukin-6 as a mediator of the effect of maternal immune activation on fetal brain development

Stephen Smith, Paul Patterson

Human studies demonstrate an increased risk for both schizophrenia and autism in children born to mothers who experienced a viral infection during pregnancy. We are exploring a mouse model in which administration of influenza virus during pregnancy induces behavioral abnormalities in the adult offspring. The virus itself is not necessary, as we find that stimulation of the maternal immune system by dsRNA (poly(I:C)) is sufficient to cause behavioral abnormalities in the offspring such as deficits in prepulse inhibition, latent inhibition and open field exploration. To test the hypothesis that elevated maternal cytokines mediate the effect of maternal immune activation on fetal brain development we administered individual cytokines to pregnant mice. A single injection of interleukin-6 (IL-6) is sufficient to cause behavioral deficits in the offspring. Moreover, administration of anti-IL-6 antibody along with poly(I:C) inhibits the effects of poly(I:C) on the offspring. Another project involves the study of maternal inflammatory cells in the fetuses of poly(I:C)-injected mothers.

141. The effect of prenatal influenza infection on neurobehavioral development of mouse offspring

Natalia Malkova, Preetha Sinha

We are investigating the neurobehavioral development of mouse pups born to mothers whose immune systems were activated at mid-gestation. Tests include the rate of ultrasound vocalizations, which is very important for mother–infant social interaction, maturation status of neuromotor reflexes, and neurogenesis. We find that injection of double-stranded RNA (poly(I:C)), which evokes an inflammatory response in the mother similar to that induced by influenza virus, alters the behavior of the offspring. Compared to controls, 10-day old C57BL/6J pups born to mothers given 10 mg/kg poly(I:C) have lower rates of ultrasound calling when separated from their mothers. We also monitored developing motor reflexes such as negative geotaxis, righting, and grasping and used body weight as an indicator of general health. No difference is found in the physical abilities and general health in the control and experimental groups. Thus, the deficit in the social behavior of pups born to mothers with activated immune systems is not due to a delay in physical development.

142. Imaging hallucinations in mice

Natalia Malkova, Doris Tse, Paul H. Patterson

We are interested in whether hallucinations can be assayed in mice. Hallucinations can be defined as the normal activation of the visual or auditory system in the absence of appropriate sensory input. A corollary is that such activity should be enhanced by drugs that are known to induce hallucinations in normal people and that exacerbate this symptom in schizophrenic subjects. Activation of 5-HT_{2A} receptors (5-HT2AR) is thought to underlie the psychomimetic properties of hallucinogenic chemicals in humans. 5-HT_{2A} receptor agonists such as 2,5-dimethoxy-4-iodoamphetamine (DOI) and lysergic acid diethylamide (LSD) stimulate head twitches in mice, which are not seen in 5-HT2AR null mutant mice. DOI and LSD stimulate similar profiles of *erg 1, erg 2* and *period 1* mRNAs [Gonzalez-Maeso *et al*. (2003) *J. Neurosci.* **23**:8836].

We find that DOI induces stereotyped behavior in mice in a dose-dependent manner, and that this drug specifically up-regulates *egr 1* and *c fos* but not *egr 2* or period-1 immunoreactivity in the auditory, visual and somatosensory cortices in a dark and quiet environment. In addition, 15 min exposure to DOI increases *c jun* phosphorylation in the somatosensory cortex and amygdala. Thus, in the absence of external acoustic and visual input, the hallucinogen DOI activates the sensory system and the amygdala, which has a primary role in the processing and memory of emotional reactions.

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143. Effects of LIF on adult neural stem cells in normal and APP23 mice

Sylvian Bauer

Although neural stem cells (NSCs) persist in various areas of the adult brain, their contribution to brain repair, after injury, is very limited. Treatment with exogenous growth factors can mitigate this limitation, suggesting that the brain environment is normally deficient in permissive cues and that it may be possible to stimulate the latent regenerative potential of endogenous progenitors with appropriate signals. We analyzed the effects of over-expressing the cytokine leukemia inhibitory factor (LIF) on adult neurogenesis in the normal brain. We found that LIF reduces neurogenesis in the olfactory bulb and subventricular zone by acting directly on NSCs. LIF appears to promote their self-renewal, preventing the emergence of more differentiated cell types. This ultimately leads to an expansion of the NSC pool. Our results have implications for the development of

therapeutic strategies for brain repair, and suggest that LIF may be useful, in combination with other factors, in promoting regeneration in the adult brain.

Adult neurogenesis is studied *in vivo* using thymidine analogues such as bromodeoxyuridine (BrdU) to label DNA synthesis during the S-phase of the cell cycle. However, BrdU may also label DNA synthesis events not directly related to cell proliferation, such as DNA repair and/or abortive reentry into the cell cycle, which can occur as part of an apoptotic process in post-mitotic neurons. We used three well-characterized models of injury-induced neuronal apoptosis, and the combined visualization of cell birth (BrdU labeling) and death (TUNEL staining) to investigate the specificity of BrdU incorporation in the adult mouse brain *in vivo*. We find that BrdU is not significantly incorporated during DNA repair, and that labeling is not detected in vulnerable or dying post-mitotic neurons, even when a high dose of BrdU is directly infused into the brain. These findings have important implications for a controversy surrounding adult neurogenesis: the connection between cell cycle reactivation and apoptosis of terminally differentiated neurons.

144. Generating mice that can be used to identify, alter or ablate newly generated neurons in the adult central nervous system

Benjamin E. Deverman

Neurogenesis persists in the adult brain and might have a role in recovery from injuries to the central nervous system (CNS). However, the study of neurogenesis is currently limited by the lack of adequate tools to identify and assess the participation of newly generated cells in functional recovery *in vivo*. To address this problem, we generated mice with genetic modifications that we hoped would allow us to permanently mark, genetically alter or ablate newly generated cells throughout the CNS at any time desired. We constructed a targeting vector that was used to insert a doxycycline (dox)-inducible Cre recombinase system into the regulatory sequences of the *sox 2* gene, a transcription factor whose expression is restricted to neural stem/progenitor cells. Unfortunately, when these mice were crossed with a Cre-dependent reporter line, and their offspring were treated with or without dox to test for dox-inducible reporter expression, reporter expression was widespread throughout the CNS in the absence of dox. While we do not yet understand the cause of this dox-independent, Sox2-independent Cre expression, one possibility that we are now investigating is that promoter interference from the PGK-neo cassette, which was inserted in the targeting vector to facilitate selection of embryonic stem cell clones carrying our knock-in cassette, causes leaky Cre expression. We have now removed this cassette from the knock-in line by crossing these mice to flp recombinase expressing mice (the neo cassette is flanked by frt sites) and are currently mating these new knock-in mice with cre-dependent reporter mice to test for dox-dependent cre expression in the offspring. If we can overcome the difficulties with leaky Cre expression, these mice will provide us with a powerful tool to: (1) analyze the contribution that newly generated neurons and glia make in the healthy and diseased/injured adult brain; and, (2) develop therapies that enhance the repair capacity of endogenous stem/progenitor cells in models of CNS disease and injury.

145. The effect of endothelin receptor antagonists on cancer cells

Jennifer Montgomery

We are investigating the effect of two distinct endothelin receptor B (ETRB) antagonists on the proliferation of melanoma and glioma cells. Previous work in this laboratory indicated that the ETRB antagonist BQ788 decreases melanoma cell proliferation *in vitro* and *in vivo.* We find that this is also true for the antagonist A-192621 *in vitro*. Moreover, both ETRB antagonists decrease proliferation in some glioma cell lines. Very high doses of antagonist are required to achieve a significant decrease in viable cell numbers, however. This, along with data on ETRB expression, led us to question the mechanism of how the ETRB antagonists are working. Preliminary evidence suggests that these antagonists may not be affecting proliferation changes through ETRB. We are further assessing this by siRNA knockdown of ETRB, as well as exploring alternative signaling possibilities by microarray analysis.

146. Effects of size and aggregation on the neurotoxicity of Mn_3O_4 nanoparticles

Diana Stefanescu, Ali Khoshnan, Janet Hering, Paul H. Patterson

Nanoparticles are used in many daily products including cosmetics and drug delivery. Exposure of humans to manganese can lead to the neurological disorder, manganism. The pathology and symptoms of the disease resemble those of Parkinson's disease. To better understand their mechanism of action, we are exploring the effects of manganese oxide nanoparticles on a human embryonic neuronal cell line. Our initial data suggest that exposure to Mn_3O_4 causes dose-dependent cell death, which is influenced by particle size and time of exposure. Moreover, exposure to Mn_3O_4 results in activation of the IKK-NF-κB, as well as the p53 signaling pathways. Studies are in progress to examine the mechanism of Mn_3O_4-induced cell death and to determine how the interaction between p53 and NF-κB may regulate neuronal death.

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Summary: Synapses, the points of contact and communication between neurons, can vary in their size, strength and number. The ability of synapses to change throughout the lifetime of the animal contributes to the ability to learn and remember. We are interested in how synapses are modified at the cellular and molecular level. We are also interested in how neuronal circuits change when synapses change their properties. We conduct all of our studies in the hippocampus, a structure known to be important for memory in both humans and animals. We use molecular biology, electrophysiology and imaging to address the questions detailed below.

A major focus of the lab concerns the cell biological mechanisms that govern modifications at individual synaptic sites. In particular, we are interested in the idea that dendritic protein synthesis and degradation may contribute to synaptic plasticity. We are also interested in mRNA and protein trafficking during synaptic plasticity.

We are also examining the role of the cadherins family of cell adhesion molecules in synaptic plasticity. Several labs have shown that cadherins are localized to synapses in the hippocampus. Earlier, we demonstrated that function-blocking cadherin antibodies or peptides can prevent long-term potentiation, without interfering with basal synaptic transmission. We hypothesize that cadherin bonds may be sensitive to local fluxes in extracellular calcium imposed by action potential activity. We are now examining the molecular mechanisms by which cadherins influence synaptic strength and the involvement of cadherins in the formation and maintenance of synapses, using fluorescence resonance energy transfer and endocytosis assays.

A relatively new endeavor in the lab involves the recording of single neuron activities in the medial temporal lobe of human epilepsy patients. In these studies, we are able to correlate single neuron responses with behavioral experience and performance. These studies should elucidate some fundamental mechanisms of brain coding and representation.

147. BONCAT and FUNCAT - novel approaches for the identification and visualization of locally synthesized proteins in neuronal dendrites

Daniela C. Dieterich[*], Jennifer J. Lee, John T. Ngo[1], David A. Tirrell[1], Johannes Graumann[2]

In both normal and pathological states, cells respond rapidly to environmental cues by synthesizing new proteins. Both the selective identification and visualization of a newly synthesized proteome has been hindered by the basic fact that all proteins, new and old, share the same pool of amino acids and thus are chemically indistinguishable. Recently, we have developed a tool (BONCAT) for the specific identification of newly synthesized proteins. This approach is based upon the co-translational introduction of small bioorthogonal chemical groups via the incorporation of modified amino acids into proteins and the chemoselective tagging of labeled proteins with an affinity tag via [3+2] click chemistry. Furthermore, we have extended this proteomic approach by using fluorescent tags to visualize newly synthesized proteins (FUNCAT).

Incorporation of the modified amino acids is unbiased, non-toxic and does not increase protein degradation. As a first demonstration of the BONCAT method, we recently reported the selective purification and identification of 195 metabolically-labeled proteins with multidimensional liquid chromatography in-line with tandem mass spectrometry (LC-MS/MS) in a mammalian cell line. The identified proteins, synthesized in a 2-hour window, possess a broad range of biochemical properties and span most functional gene ontology categories. The mass spectrometrical identification of newly synthesized proteins is performed in the laboratory of Professor Ray Deshaies, Division of Biology, at Caltech. In addition to BONCAT, we are now able to visualize newly synthesized proteins *in situ* in primary hippocampal neurons using water-soluble fluorescent tags (FUNCAT) by confocal microscopy. While abundant signal is detected in cells treated with the modified amino acids, no signal is detected in protein synthesis-inhibitor treated cultures, confirming that this procedure labels newly synthesized proteins with high specificity. Strikingly, newly synthesized proteins in the soma of dissociated hippocampal cultures can be detected as early as 10 minutes after contact with the modified amino acids. This new set of tools makes it possible to address the temporal

and spatial characteristics of newly synthesized proteomes in any cell type under basal, as well as, elevated levels of synaptic activity.

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148. Elucidating the hippocampal dopaminergic proteome using bio-orthogonal noncanonical amino acid tagging (BONCAT)

Jennifer J. Lee, Daniela C. Dieterich[*], David A. Tirrell[1]

Dopamine is a crucial neurotransmitter that innervates multiple structures in the brain, such as the striatum, prefrontal cortex, and the hippocampus. Abnormalities in dopamine regulation are responsible for disorders like Parkinson's disease and schizophrenia. Functional magnetic resonance imaging (fMRI) studies found that there is activation in the mesolimbic system during reward-mediated learning. The mesolimbic system is one of the four major dopaminergic pathways in the human brain and includes the ventral tegmental area and the hippocampus. The hippocampus is a bilateral, horn-shaped structure that has been implicated in the establishment of learning and memory in mammalian species ranging from rats to humans.

Recent work in cultured hippocampal neurons by our lab has shown that dopamine modulates local protein synthesis in neuronal dendrites. Even though we understand that dopamine plays a role in protein synthesis, no one has yet identified the specific proteins that are produced upon dopaminergic stimulation. In order to specifically enrich for the newly synthesized proteins produced upon dopaminergic stimulation, we have employed the above-described approach of bio-orthogonal noncanonical amino acid tagging (BONCAT). Using this, in conjunction with multidimensional protein identification technology (MudPIT), we are able to identify the proteins in synaptoneurosomes in which synthesis is modulated by dopamine with high levels of accuracy and sensitivity. The mass spectrometrical identification of newly synthesized proteins is performed in the laboratory of Professor Raymond J. Deshaies, Division of Biology, at Caltech. The mass spectrometry data is then passed through the Trans-Proteomic Pipeline (TPP, developed by the Institute for Systems Biology in Seattle, WA), which provides tools for quantification and identification of our proteins. To identify the dopamine-induced somatic proteome, we analyze newly synthesized proteins from whole dissociated hippocampal cultures and acute hippocampal slices; for the dendritic proteome, we analyze newly synthesized proteins from rat brain synaptoneurosomes and isolated dendrites of hippocampal cultures. Synaptoneurosomes are a biochemical fraction enriched with translation-active synaptic terminals, but devoid of somata and nuclei. Isolated dendrites will be obtained from a special culture system using polycarbonate nets to separate dendrites from cell bodies.

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149. Regulation of spine morphological dynamics and synaptic function by N-cadherin

Shreesh P. Mysore, Chin Yin Tai

N-cadherin is a cell adhesion molecule that has been shown to play an important role in synapse formation during the development of neuronal circuits. However, the extent of its role in the maintenance of mature synapses is largely unknown. We investigated the role of N-cadherin in regulating the structure and function of mature hippocampal synapses in cultured neurons. We found that the acute disruption of N-cadherin with a 10-minute application of the HAV peptide produced drastic effects on synaptic structure. Time-lapse confocal microscopy revealed that spines shorten and are more motile soon after N-cadherin disruption (75 minutes post treatment). This destabilization of spines is followed by spine loss at 180 minutes post-treatment. We find that the earlier increase in motility and decrease in length are predictive of later spine loss. In addition to structural postsynaptic changes, we find changes both in the function and the biochemical composition of synapses. The frequency of miniature synaptic events is reduced to 50% at 30 minutes following N-cadherin disruption. Concomitantly, we see a decreased binding of N-cadherin to β-catenin, a signaling molecule immediately downstream of N-cadherin. Consistent with earlier work, we see an increase in the tyrosine phosphorylation of β-catenin as well. Experiments in heterologous cells suggest that these large-scale synaptic changes are the result of a long-lasting effect of the HAV peptide on N-cadherin mediated adhesion. Thus, our work sheds light on the key role of N-cadherin in the stability and function of mature synapses.

150. Information integration in CA1 pyramidal neurons

Hiroshi Ito

A number of studies indicate that the hippocampus is required to form certain types of memory. Among the subregions of the hippocampus, area CA1 is considered as a final relay station of the hippocampal formation and thus is expected to play a significant role in hippocampal function. Area CA1 receives two distinct excitatory synaptic inputs; one is from area CA3 (Schaffer-collateral pathway) and the other is from the entorhinal cortex (temporoammonic pathway). Considering the large differences in *in vivo* neural activities between area CA3 and the entorhinal cortex, information integration between two pathways is likely crucial for hippocampal function. We are interested in the modulation of integration by several types of neuromodulators, such as dopamine or acetylcholine, which are thought to have important roles in learning. We observe that dopamine not only depresses

basal synaptic transmission, but also enhances long-term potentiation (LTP) in the temporoammonic pathway. However, under GABA receptors blockade with bicuculline and CGP55845, dopamine still depresses basal synaptic transmission but does not enhance LTP, which suggests that interneurons play a major role in dopamine-induced enhancement of LTP. We hypothesize that the dopamine-induced depression of excitatory inputs to interneurons reduces inhibition of pyramidal neurons, which eventually enhances LTP. Furthermore, our data suggests that the impact of dopaminergic modulation depends on the stimulus frequency; i.e., higher frequency stimuli have a larger impact under dopamine application. On the other hand, in spite of strong modulation of the temporoammonic pathway, we did not observe major effects of dopamine on the Schaffer-collateral pathway. Considering our results together, dopamine selectively modulates the impact of the temporoammonic pathway and regulates the information flow in area CA1.

151. Activity-dependent dynamics and sequestration of proteasomes in dendritic spines

Baris Bingol, Erin M. Schuman

The regulated degradation of proteins by the ubiquitin proteasome pathway is emerging as an important modulator of synaptic function and plasticity. The proteasome is a large, multi-subunit cellular machine that recognizes, unfolds and degrades target polyubiquitinated proteins. Here we report the NMDA (N-methyl-Daspartate) receptor-dependent redistribution of proteasomes from dendritic shafts to synaptic spines upon synaptic stimulation, providing a mechanism for local protein degradation. Using a proteasome-activity reporter and local perfusion, we show that synaptic stimulation regulates proteasome activity locally in the dendrites. We used restricted photobleaching of individual spines and dendritic shafts to reveal the dynamics that underlie proteasome sequestration, and show that activity modestly enhances the entry rate of proteasomes into spines while dramatically reducing their exit rate. Proteasome sequestration is persistent, reflecting an association with the actin-based cytoskeleton. Together, our data indicate that synaptic activity can promote the recruitment and sequestration of proteasomes to locally remodel the protein composition of synapses.

152. eEF2 couples weak synaptic activation to suppression of dendritic translation

Michael A. Sutton, Erin M. Schuman

Local protein synthesis in neuronal dendrites has emerged as a powerful mechanism for regulating both basal synaptic function and activity-dependent changes in synaptic efficacy. We have previously reported that ongoing weak synaptic activation conferred by miniature synaptic transmission (minis) inhibits local translation in dendrites of hippocampal neurons (Sutton *et al*., 2004) and that this regulation acts to stabilize synaptic function during activity blockade (Sutton *et al*., 2006). Here, we show that the coupling of minis to the translational machinery in dendrites is mediated, in part, by local regulation of eukaryotic elongation factor (eEF2). eEF2 catalyzes ribosomal translocation during polypeptide elongation, and its activity is strongly inhibited by phosphorylation. In the absence of action potential (AP)-evoked neurotransmission, mini blockade inhibits eEF2 phosphorylation, whereas acute stimulation of mini frequency enhances eEF2 phosphorylation. Consistent with a role for mini-induced eEF2 phosphorylation in mediating translational suppression, pharmacological disruption of eEF2 kinase stimulates dendritic synthesis of a fluorescent translation reporter, and restricted perfusion of eEF2 kinase inhibitors produces local translational activation in treated dendritic segments. These results thus indicate that eEF2 serves as a local biochemical sensor designed for coupling weak synaptic activation with local translational suppression in dendrites.

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153. β-catenin regulates the NMDAR-dependent N-cadherin internalization at excitatory synapses

Chin Yin Tai, Shreesh P. Mysore

N-cadherin, one of the major cell adhesion molecule at excitatory synapses, regulates synaptic structure and plasticity. The amount of N-cadherin present at synapses regulates the strength of the adhesive force across the synaptic junction. Very little is known, however, about the turnover of N-cadherin at the synapse and the mechanisms that underlie its exo- and endocytosis. Our results show, for the first time, that the constitutive internalization and recycling of N-cadherin occurs rapidly. We hypothesized that this turnover can be regulated by synaptic activity. Indeed, we found that the activation of NMDAR causes a delay in the N-cadherin internalization. This delay resulted in an accumulation of surface N-cadherin at both synaptic- and extrasynaptic sites. To elucidate the mechanism, we investigated the role of β-catenin, an N-cadherin binding protein, in the regulation of N-cadherin endocytosis. In heterologous cells, β-catenin negatively regulates the endocytosis of N-cadherin. In neurons, NMDAR activity resulted in an increase in the N-cadherin/β-catenin binding accompanied by a decrease in the amount of tyrosine phosphorylated β-catenin. These results suggest that the tyrosine phosphorylation of β-catenin regulates N-cadherin endocytosis. When a mutant form of β-catenin (Y654F, mimics no phosphorylation at tyrosine residue 654) was expressed in neurons, the NMDAR-dependent regulation of N-cadherin internalization was abolished and surface N-cadherin was stabilized, suggesting that tyrosine residue 654 of β-catenin is required for this process. In addition,

we found that the overexpression of β-catenin Y654F mutant in neurons blocks chemical long-term depression (chemLTD) produced by NMDA treatment. Together, we have demonstrated that NMDAR activity regulates N-cadherin internalization by mediating the tyrosine phosphorylation of β-catenin. We have also demonstrated that the prolonged stabilization of N-cadherin at the surface causes deregulation of synaptic plasticity. We propose that the β-catenin-dependent regulation of N-cadherin endocytosis plays an important role not only in structural plasticity but also in synaptic function.

154. Delivery of RNAs into neurons using the penetratin peptide

Hwan Ching Tai

We are interested in utilizing the penetratin peptide to develop new tools to study synaptic function in neurons. Penetratin is a 16-mer peptide sequence found in the homeodomain of *Drosophila Antennapedia* protein. Penetratin is capable of crossing the cytoplasmic membrane by clathrin-independent endocytosis. It has been shown to function in essentially all mammalian cells tested to date, including neurons. Penetratin can be utilized to carry various types of molecular cargos across the cell membrane, if the cargo is covalently linked to penetratin. When the covalent linkage is a disulfide bond, the cargo can be automatically released inside the cell due to reduction by intracellular glutathione. Penetratin has been successfully applied to deliver small molecules, as well as, macromolecules such as DNAs, RNAs and peptides.

Our laboratory studies synaptic function in hippocampal neurons. In hippocampal neurons, protein-synthesis-dependent forms of synaptic plasticity can occur on the timescale of tens of minutes to several hours. Therefore, fast-acting RNA interference (RNAi) methods are required to investigate such dynamic changes. RNAi in cultured neurons is generally based on DNA-derived siRNAs, which are slow-acting because vector entry and gene transcription are rate-limiting steps. We are testing if penetratin-mediated siRNA could induce fast-acting RNAi responses. Recent reports have shown that microRNAs and enzymes required for microRNA function (Dicer and Argonaute) are localized in dendrites. We are also interested in delivering microRNAs via penetratin to examine the role of microRNAs in the dynamic regulation of synaptic function.

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155. Visualization of cadherin-cadherin association in living cells

Eric Mosser

In the last decade, much effort has been devoted to understanding the structure and molecular associations of classic cadherins. Crystallographic and biophysical studies have yielded somewhat conflicting results and an, as yet unclear, picture of the homotypic interactions of the cadherin extracellular domain during dimerization. To better understand the dynamics of cadherin interactions we have developed Fluorescence Resonance Energy Transfer (FRET)-based sensors to monitor cadherin associations across cellular junctions in a dynamic manner in living cells.

We have fluorescently tagged cadherin molecules in their extracellular domains and expressed FRET-donor and FRET-acceptor cadherins in pre- and postjunctional cells to monitor the strength of adhesion. We have demonstrated FRET between ECFP and EYFP labeled cadherins in double-transfected HEK 293 cells (expressing both donor and acceptor constructs), as well as, FRET between adjacent single-transfected COS7 cells.

It has long been known that the structural integrity of cadherins and their dimerization status is dependent on the local calcium concentration. We have observed that reducing Ca^{2+} in the extracellular solution leads to a significant loss of FRET signal at COS7 cell junctions expressing FRET pairs and have used repeated, fast scans of cell pairs to monitor FRET changes dues to changes in extracellular Ca^{2+} during perfusion with Ca^{2+} or Ca^{2+} chelators; allowing us to examine the dynamics of cadherin-cadherin interactions on a shorter time scale.

FRET is unique in its ability to provide signals that are sensitive to changes in intra- or intermolecular distances in the 1-10 nm range, well below the inherent diffraction limit of conventional fluorescence microscopy. We believe that FRET is a powerful technique to monitor cadherin orientation and interactions in heterologous, living cells.

156. Stimulation-dependent assembly and trafficking of ribosomes in neurons

Young J. Yoon

Previous observations of ribosomes in dendrites of hippocampal neurons suggests that the translational capacity of synapses in spines is limited by the number of ribosomes available in the dendritic pool. Studies of fixed samples have shown that each synapse, on the average, possesses only one to two polysomes and furthermore, only 40% of spines have polysomes after induction of plasticity by high-frequency stimulation. The apparent shortage of translational machinery in dendrites poses several challenges to cells that utilize local translation as a key mechanism to modulate cellular responses to a multitude of external stimuli. To this end, experiments have been designed to address the ways in which neurons may budget or expand their translational capacity to meet the growing demands. Our goal is to test and determine whether neurons manage the translational capacity of its

dendrites in a dynamic (shared) or static (all or none) manner.

There have been successes in studying ribosomes in cultured cells by expressing fluorescently-labeled ribosomal proteins. By expressing 60S and 40S subunit proteins which are tagged with different fluorescent proteins, it will be possible to track localization of the subunits by time-lapse imaging. In addition, it will be possible to study stimulation-dependent trafficking and assembly of ribosomes as neurons are cells with high morphological complexity.

157. Firing properties of human hippocampal neurons during reward expectance and delivery

Ueli Rutishauser, Adam N. Mamelak

The projections of midbrain dopaminergic neurons to cortex and the limbic system are crucial for many forms of learning. Dopaminergic neurons fire when animals encounter unexpected events (novelty) or when there is a mismatch between what (reward) is expected and what is received. The hippocampus is known to be crucially involved in learning and receives a prominent dopaminergic input. Recently, we have identified neurons in the human hippocampus that fire strongly only to the first ("novelty detectors") or second and following ("familiarity detectors") presentation of a stimulus. These neurons are thus capable of single-trial learning. We have thus constructed a behavioral task involving reward to investigate whether dopamine modulates the firing properties of hippocampal neurons. We recorded single neuron activity from intracranial depth electrodes implanted in the medial temporal lobe (MTL) and anterior cingulate cortex of epilepsy surgery patients. The patients were exposed to a demanding reaction time task combined with a reward delivery. Within a given trial, visual cues were provided that predicted whether a given trial would be rewarded or not (dependent on performance). We found that a subset of human MTL neurons, both in the amygdala and hippocampus, are strongly driven by the expectance and/or delivery of reward. Particularly, we find neurons that appear to be tuned to the magnitude and valence of the reward, for example, "high reward" or "punishment." Remarkably, many neurons fire most strongly at the conclusion of a trial, following reward delivery or absence. These data suggest that the representation of learned information in the firing activity of hippocampal neurons is influenced by reward expectancy and magnitude.

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Summary: We continue to examine the dynamic/adaptive nature of human visual perception – including its crossmodal, representational, sensory-motor, developmental, emotional, and neurophysiological aspects. Meanwhile, the ERATO (Exploratory Research for Advanced Technology) Shimojo "Implicit Brain Functions" project (supported by JST, Japan Science and Technology Corporation, officially started in October '04) is fully operational now, with its emphasis on implicit cognitive processes, emotional decision, and their neural correlates. Vigorous collaborations are conducted in our psychophysics laboratory here and the Japan site located at NTT Communication Science Laboratories, Atsugi, Kanagawa, Japan.

Using a variety of methods, including eye tracking, EEG, fMRI and MEG, we examine how exactly peripheral sensory stimuli, neural activity in the sensory cortex, and the mental experience of perception are related to each other. As for objectives of the new ERATO project, we aim to understand implicit, as opposed to explicit or conscious, somatic and neural processes that lead to, and thus predict, conscious emotional decision such as preference.

In addition, we have some purely theoretical meta-analysis on human color perception, complexity of visual letters and objects, etc.

(1) We continue our work applying TMS (Transcranial Magnetic Stimulation) to the visual cortex of alert normal subjects, to reveal neural mechanisms underlying conscious visual perceptual experience. In the latest study, we have demonstrated that "impossible space" is represented in the visual cortex. We compared spatial position/extension of phosphene (illusory light field) triggered by dual-pulse TMS, when gaze upper most vs. lower most. The results strongly indicate that the phosphene may extend into space beyond the typical outer range of the visibility (such as "under the chin" or "inside of the chest"). This may be understood as an emergent property of gain field, or gaze-dependent modulation of neuronal activity. In yet another study we applied TMS to saccade mislocalization phenomenon, and provided the first direct evidence against any account based on the neural delay from the retina to the cortex. We showed that TMS-triggered phosphene, which would not have the neural delay that visual stimuli typically have, yet undergoes an equally large magnitude of saccade mislocalization.

(2) We compared color modulation of bare skin color which signals emotional change, and sensitivity of the human color system in the relevant middle-wavelength range. We then came up with a surprising conclusion that evolution of primate color perception is at least under influence of socio-emotional signaling via skin color modulation in the others.

(3) We compared the structures of letters and symbols in natural languages on one hand, and those of objects in natural scenes on the other hand. It turned out that the structures of letters and symbols throughout human history are selected to match those found in objects in natural scenes.

(4) Applying adaptation/aftereffect paradigm and fMRI, we investigate how consciously visible/invisible adaptor contributes to aftereffect. In the special case of face adaptation, unlike lower-level perceptual adaptations such as tilt or contrast adaptation, we found that only visible duration of adaptor face contributes to face-identity aftereffect later. On a related issue, when the eye re-fixated elsewhere after adaptation, there was a gaze-dependent modulation of color and depth aftereffects. The effect of face adaptation seems to be spatially un-tuned, that is, remains everywhere regardless of the location on the retina or the space.

(5) Following our earlier work indicating that dynamic gaze shift (and perhaps orienting response in general) is a somatic precursor of conscious preference judgment, we extended the analyses by manipulating the initial attractiveness difference in the pair of faces, and see how much gaze manipulation can affect the preference decision depending on the initial differences. The result indicates that even with the largest baseline difference, gaze manipulation affects the final preference decision equally effectively.

(6) We compared reward and punishment-avoidance in fMRI. While obtaining a reward-elicited activity in classical reward structures, avoidance of punishment did not elicit significant activity in those areas. These data suggest that reward and avoidance conditioning are very distinct processes despite their common behavior-reinforcing properties.

(7) As a part of the ERATO project, we examined fMRI signals in response to a more preferable face vs. a less preferable one. As a result, we found some early activation of a subcortical area (nucleus accumbens) with late activation of a more prefrontal area. This early activation was task-independent, thus indicating automatic, bottom-up emotional activation.

(8) We continue to address the issue of how position and motion information are linked in the visual processing. In a psychophysical study, we examined how sequence-of-snapshots evolve to a smooth motion trajectory. Yet another study examined contradictory perception of position and motion trajectory. The third study demonstrated a cyclopean (i.e., binocularly fused) version of the flash lag.

(9) In collaboration with Boston University group, we examine a very paradoxical situation where weaker visual motion signals (distractor) lead to a larger interference effect with motor responses. We suspect that this may be something to do with salience of the distractor signals and top-down control specific to the task.

(10) We reported a special case of auditory-visual integration where visual motion effect sound source localization (ventriloquism) except that the effect is cumulative over cycles. We coined it "moving ventriloquism."

158. The optimal human ventral stream from estimates of the complexity of visual objects

Mark A. Changizi

The part of the primate visual cortex responsible for the recognition of objects is parceled into about a dozen areas organized somewhat hierarchically (the region is called the ventral stream). Why are there approximately this many hierarchical levels for object recognition? Here I put forth a generic information-processing hierarchical model for visual object recognition, and show how the total number of neurons required depends on the number of hierarchical levels and the complexity of visual objects that must be recognized. Because the recognition of written words appears to occur in a similar part of inferotemporal cortex as other visual objects, the complexity of written words may be similar to that of other visual objects; for this reason, I measure the complexity of written words, and use it as an approximate estimate of the complexity of visual objects more generally. I then show that the information-processing hierarchy that accommodates visual objects of that complexity possesses the minimum number of neurons when the number of hierarchical levels is approximately 15 and when the sizes of areas decrease exponentially with level, each level on average approximately 1.25 times larger than the level above it. I show that these optimal properties are close to those found in the primate ventral stream.

159. Bare skin, blood, and the evolution of primate color vision

Mark A. Changizi, Qiong Zhang, Shinsuke Shimojo

The primate face undergoes color modulations (such as blushing or blanching), some which may be selected for signaling and some that may be an inevitable consequence of underlying physiological modulations. Because for highly social animals like most primates, one of the most important kinds of object to be competent at perceiving and discriminating is other members of one's own species, we hypothesized that primate color vision has been selected for discriminating the short-term spectral modulations on the skin of conspecifics, these modulations providing useful information about the current state or mood of another conspecific. Here we show that for the two dimensions of skin spectral variation in the short term, the dimension due to the fraction of blood in the skin corresponds approximately to the blue-yellow opponent channel (more blood ==> bluer), and the other dimension due to oxygen saturation of the blood corresponds approximately to the red-green opponent channel (greater oxygenation ==> redder). Trichromats, but not dichromats, are therefore, sensitive to both dimensions of skin color variation, and, more specifically, the wavelength sensitivities of the M and L cones for trichromatic primates are near optimal for sensing modulations of oxygen saturation. Also, because skin color modulation cannot be seen on a furry face, trichromatic primates tend to have bare faces.

160. The structures of letters and symbols throughout human history are selected to match those found in objects in natural scenes

Mark A. Changizi, Qiong Zhang, Hao Ye, Shinsuke Shimojo

Are there empirical regularities in the shapes of letters and other human visual signs, and if so, what are the selection pressures underlying these regularities? To examine this, we determined a wide variety of topologically distinct contour configurations, and examined the relative frequency of these configuration types across non-logographic writing systems, Chinese writing, and non-linguistic symbols. Our first, and main, result is that these three classes of human visual sign possess a similar signature in their configuration distribution, suggesting that there are underlying principles governing the shapes of human visual signs. Second, we provide evidence that the shapes of visual signs are selected to be easily seen, at the expense of the motor system. Finally, we provide evidence to support an ecological hypothesis that visual signs have been culturally selected to match the kinds of conglomerations of contours found in natural scenes, because that is what we have evolved to be good at visually processing.

161. Does avoiding a punishment act as a reward? An fMRI study of avoidance learning

Hackjin Kim[*], Shinsuke Shimojo, Ralph Adolphs[*], John P.O'Doherty[*]

Avoidance conditioning involves learning to perform a response to avoid an aversive event. Avoidance learning poses a challenge for theories of instrumental conditioning because once the punishment is successfully avoided the subject no longer experiences negative reinforcement for their behavior. Thus, behavior appears to be maintained in the absence of reinforcement. One possible account for this is to propose that avoiding a punishment is in itself a reward, and thus, avoidance behavior is positively reinforced on each trial when the punishment is successfully avoided. In the present study we aimed to address the question of whether avoidance of punishment recruits the same or distinct neural circuitry as that elicited by a reward itself. We scanned 16 human subjects with fMRI while they performed an instrumental choice task in which they could win or lose money. In reward condition subjects could choose one of two actions that led to a greater (60%) or lesser (30%) probability of obtaining a monetary reward (gaining $1). In avoidance condition they could choose between two actions which were associated with a greater (60%) or lesser (30%) chance of avoiding a monetary loss (losing $1). Over the course of the experiment subjects showed a statistically significant preference for the action associated with a lesser probability of punishment and for the action associated with the greater probability of reward, indicating that they had shown avoidance, as well as reward conditioning. However, contrary to the reinforcement account of avoidance learning, avoiding a punishment and obtaining a reward were associated with different patterns of neural activity. While obtaining a reward-elicited activity in classical reward structures such as medial orbitofrontal and adjacent medial prefrontal cortices, ventral striatum, midbrain and bilateral amygdala, avoidance of punishment did not elicit significant activity in those areas. These data suggest that reward and avoidance conditioning are very distinct processes despite their common behavior-reinforcing properties.

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Citation

Kim, H., Shimojo, S., Adolphs, R. and O'Doherty, J.P. (2005) Program No. 74.8, Abstract Viewer/Itinerary Planner. Washington, DC: Society for Neuroscience, online.

162. Auditory-motor delay adaptation modulates subjective simultaneity of visually observed other's action and auditory stimuli

Masataka Watanabe[*], Shion Shinohara[*], Shinsuke Shimojo

Prolonged exposure to biased sensorimotor relationship leads to recalibration of the two modalities, spatially (Welch, 1978) and/or temporally (Cunningham *et al*., 2001). We question if personal sensorimotor adaptation modulates the perception of others performing identical motor actions with sensory feedback.

The experiment consisted of three phases: Pretest, adaptation and post-test. In the adaptation phase, subjects were exposed to temporal misalignment of auditory stimulus and arm motion. Subjects moved a computer mouse horizontally while a delayed (150ms) "click" sound was delivered whenever the mouse ceased to move. We instructed the subjects to temporally align the "click" to a metronome "beep." Only a fixation spot was displayed on the monitor and the subject's arm was screened to eliminate possible auditory-visual adaptation.

For the test of adaptation, we examined three types of subjective simultaneity on an event basis without the use of a metronome. In the "self" test, the participants judged the simultaneity of self-mouse stoppage and a "click" sound. In the "other" test, the participants viewed the experimenter maneuvering the mouse and judged the simultaneity of mouse stoppage and a "click." Finally for control, we tested the subjective simultaneity of a simple visual flash and a "click."

The psychophysical results indicated statistically significant shifts in subjective simultaneity toward the lag of auditory stimulus for both the "self" test (+72 ms) and the "other" test (+35 ms), but not for control. Our results suggest possible involvement of the mirror system in projecting personal sensorimotor recalibration to observation of other's action.

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163. A systematic investigation of the gaze manipulation effect

Claudiu Simion, Shinsuke Shimojo

We (Shimojo, Simion *et al*, 2003) have advanced the hypothesis that orienting behavior and cognition interact in a positive feedback loop forming the backbone of preference decisions. The fewer the cognitive "reasons" to prefer a stimulus in a comparison task, the stronger the influence of orienting behavior. We demonstrated that orienting can directly influence subjects' preference decision by manipulating subjects' gaze, and that the stimuli oriented towards for longer times were chosen as more attractive when the difference in base attractiveness rating was small. The effect was not due to mere exposure. The present study is a systematic investigation of the phenomenon of gaze manipulation under various cognitive loads. Observers rated a human face database for attractiveness, after which they were presented with pairs of faces drawn from this database. The pairing was done so that the difference in attractiveness rating varies from large to zero. One of the faces was the “target," and the subject was forced to orient to it longer. We show that, regardless of the initial difference in rating, the percentage of cases in which the target face is chosen is on-average 14 percent larger in the gaze manipulation compared to the control task. We conclude that orienting influences preference at all levels of cognitive load, making a stimulus initially considered less attractive more likely to be preferred.

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Citation

Simion, C. and Shimojo, S. (2006) *J. Vision* 6(6), 282a, http://journalofvision.org/6/6/282/ doi:10.1167/6.6.282.

164. Perisaccadic localization of TMS-induced phosphene

Junghyun Park, Daw An Wu, Shinsuke Shimojo

Accurate spatial localization of a visual stimulus requires the integration of its retinal position with the direction of gaze at the time of stimulus presentation. But studies have shown that brief visual targets presented near the time of saccades tend to be mislocalized. Mislocalization starts growing long before a saccade (~100ms) and reaches a maximum at saccade onset. This presaccadic mislocalization has often been attributed to the temporal mismatch between the visual and eye position signals due to the long visual afferent delay (>50ms). To directly test if the visual afferent delay is necessary for presaccadic mislocalization, we investigated how observers localize phosphenes induced by transcranial magnetic stimulation (TMS) of the visual cortex (thus, bypassing the afferent delay) around the time of saccades.

Observers who consistently perceived single phosphene with a sharp vertical edge were screened and tested in two conditions. In one condition, a dual-pulse TMS was applied over the left visual cortex at various times while they were performing a delayed (rightward) saccade task. Observers reported the perceived position of the leftmost edge of the phosphene with a mouse cursor presented after a saccade. In the other condition, instead of applying TMS, a dim vertical bar was presented (13ms) on the monitor screen.

Most observers mislocalized TMS-induced phosphene near the time of saccades as they did for the flashed bar. The time course and magnitude of the error for both types of stimuli were practically identical. Thus, presaccadic mislocalization should be explained with some other factors than afferent delay.

Citation

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165. Face adaptation depends on gaze (overt attention) to the face

Farshad Moradi, Shinsuke Shimojo

We are studying gaze dependence of adaptation to faces and scenes using fMRI. Previous studies indicate face adaptation is non-retinotopic, but its gaze dependency has not been systematically examined. In Experiment 1 we demonstrated robust adaptation to foveal faces and scenes in FFA and PPA, respectively, using a psychophysical paradigm known to induce identity aftereffect: After 4 sec adaptation to a face (scene) followed by 0.8 sec blank, either a different, or an identical image of the same category (probe) was presented for 0.8 sec. Observers reported if adaptor and test were the same. A different probe evoked significantly larger BOLD response in FFA(PPA) than the same image. In Experiment 2, adaptor and probe were displayed in periphery (7.2 deg, same hemifield), and we measured BOLD activity in the contralateral area. Adaptation to faces, but not scenes, was considerably weaker than in Experiment 1. Experiment 3 examined whether gaze affects the adaptation phase, or the subsequent testing (probe). A foveal adaptor resulted in significant aftereffect in periphery. Almost no adaptation occurred when adaptor was peripheral and probe was foveal. No such dependency was found for scenes. The BOLD activities evoked by peripheral and foveal faces/scenes were about the same. Thus, different activation is ruled out as a confound. We conclude that adaptation to faces but not scenes depends on overt attention during adaptation. These results are unlikely to be due to the reduced resolution in the periphery as scenes contained more high-spatial frequency information than faces.

Citation

Moradi, F. and Shimojo, S. (2006) *J. Vision* 6(6) 875a, http://journalofvision.org/6/6/875/ doi:10.1167/6.6.875.

166. Stimulating "impossible" visual space with TMS

Daw An Wu, Junghyun Park, Shinsuke Shimojo

When peering through binoculars, one is acutely aware of the blackness that frames the field of view. One might expect that such a blackness should also frame our everyday field of vision; as we move our eyes about, portions of the retina are alternately exposed to the world or shaded by the eye socket.

Demonstration: With your gaze fixated on a distant object, tip your head downwards. As your forehead begins to descend over your field of view, you might theoretically expect to see a tall curtain of blackness over the covered area. Instead, you never see more than a thin dark band—areas beyond that simply fall out of your visual awareness.

These areas have been termed "impossible" space, because the retina cannot receive line-of-sight activation from these directions. Hayhoe & Williams (1984) and Cavanagh & Barton (VSS 2001) used afterimages and scleral illumination to demonstrate that even active retinal input corresponding to impossible locations does not reach visual awareness.

Here, we directly stimulate visual cortex with transcranial magnetic stimulation (TMS), inducing percepts of brightness (phosphenes). Because phosphenes can be induced in a retinotopically stable fashion, we can attempt to induce them in impossible locations by manipulating eye position. Participants were tested both in total darkness and under low illumination. They reported the spatial position and extent of their phosphenes with respect to various fixation points.

Almost all participants reported one or both of the following: 1) In the dark, phosphenes could extend into impossible locations. These locations were described as "inside my cheeks/chest," "behind my ears," etc. 2) Under dim light, these percepts were abolished. Participants reported shifts in location or "squashing" of the shape to fit within the visual field.

This indicates that it is possible for visual awareness to extend into ecologically impossible space. However, when environmental illumination is present, the phosphene's retinotopic-egocentric correspondences may become remapped to respect visual field boundaries.

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167. Dynamic evolution of motion perception

Bhavin Sheth[*], Ryota Kanai, Shinsuke Shimojo

In visual perception, position and motion interact reciprocally. While motion is a construct consisting of a series of sequential changes in position, the perception of motion is considered to be more than just a series of changes in the perceived stimulus position. To investigate the evolving process of motion percept starting from the onset of motion, we had observers detect a small transient gap (13 ms long) within an otherwise smooth motion sequence. Observers were highly sensitive to a gap that occurred early in the motion (< 200 ms from motion onset). However, their sensitivity to detect the gap deteriorated as the motion continued (> 300 ms), indicating perceived motion became smoother and filled in over the course of motion. Moreover, the same temporal pattern of decline in detection performance was observed for transient change in shape or color. Experiments suggest that motion blur does not account for the effect, as blur has different temporal characteristics, and blur decreases, not increases with time. Our results together imply that the visual system processes a moving stimulus initially as a series of snapshots, but then gradually develops a holistic percept of motion as the motion continues. Once the motion system is sufficiently activated, stimuli presented at different times begin to be integrated into a single, coherent entity. Our results therefore, suggest perception of object motion begins as a series of discrete, unintegrated snapshots in a way akin to that of an akinetopsic patient.

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Citation

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168. Greater response conflict from weaker visual signals

Yuko Yotsumoto[1,2], Aaron Seitz[1], Yuka Sasaki[2], Shinsuke Shimojo, Toshimasa Yamamoto[3], Masao Kogure[3], Masamichi Sakagami[4], Takeo Watanabe[1]

It is generally thought that strong conflicting signals have greater negative impacts on task performance than relatively weaker conflicting signal. Here we show that this is not necessarily the case for visual-motor contingencies. We used a novel procedure where subjects judged the color (blue or green) of a visual stimulus by making a color-contingent leftward or rightward motion. In the background, a certain percent of coherently moving dots was presented with leftward or rightward direction, but which was irrelevant to the task. In half the trials, the direction of the "correct" motor response was the same as the direction of motion-stimulus (i.e., congruent trials), whereas in the other half of trials, the correct response direction was opposite to the coherent motion direction (i.e., incongruent trials). The coherent motion percentage was randomly chosen for each trial and ranged from 0-100%. Interestingly, we found a non-monotonic relationship with a significant dip in performance at around 25% motion coherence with recoveries of performance on either side. Reaction times trended higher at 25% coherence, opposite to the prediction of a reaction-time/accuracy trade-off. The generality of this effect was confirmed with tasks using different motor responses, such as saccadic eye movements and steering wheel operations. These results are at odds with the model that stronger

irrelevant signals more severely interfere with a task performance. One possible explanation is that visual signals that are too weak to be "noticed" and suppressed can directly interfere with a motor response as conflicting motor signals.

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Citation

Yotsumoto, Y., Seitz, A., Sasaki, Y., Shimojo, S., Yamamoto, T., Kogure, M., Sakagami, M. and Watanabe, T. (2006) J. *Vision* 6(6) 791a, http://journalofvision.org/6/6/791/ doi:10.1167/6.6.791.

169. Mutually contradictory percepts in motion processing

Dylan R. Nieman, Bhavin R. Sheth[*]*, Shinsuke Shimojo*

Studies of thinking and reasoning processes have shown that people can maintain multiple, fundamentally incompatible beliefs, particularly if attention is not drawn to the incompatibility. Recent work suggests the same may be true for multiple low-level visual percepts: Whitney and Cavanagh (2000) inferred that while a target's perceived position is shifted by an inducer, there is no accompanying percept of target motion. A more explicit perceptual paradox can be shown if the motion and position percepts of a target stimulus are each tested and found to be inconsistent under conditions without an inducer. Observers (n=12) watched a small, circular target move diagonally at constant speed (21.2 deg/s) on a screen for 200 ms, then make an abrupt 90 degree turn and continue at the same speed for 200 ms. Observers fixated a small dot outside the target's path and judged the position of the turn-point relative to nearby horizontal or vertical hash marks. The two-dimensional position of the turn was systematically mislocalized to a position outside the object's motion trajectory. Our control data indicates that known effects like Frohlich, flash-lag, representational momentum, and onset repulsion are unable to explain the shift. Specific probing of the target's trajectory suggests that observers maintain no less than three percepts that are mutually inconsistent: The position of the pre-turn path, the position of the post-turn path, and the position of the turn-point itself. Our findings support the idea that lower-level sensory processing can be mutually contradictory, suggesting some module-like structure.

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Citation

Nieman, D.R., Sheth, B.R. and Shimojo, S. (2006) *J. Vision* 6(6) 554a, http://journalofvision.org/6/6/554/, doi:10.1167/6.6.554.

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17. Moving ventriloquism: Forward drifts and sharp resets in perceived audio-visual simultaneity

Shinsuke Shimojo, Ryota Kanai, Bhavin Sheth[*]

Attention is known to modulate sensory inputs; however, little is known on how the interactions between top-down and bottom-up cues dynamically evolve. Here, we show that auditory-visual perception changes systematically and non-monotonically from an interaction of sensory and attentional factors during the continuous viewing of a physically invariant stimulus. The stimulus was a visual target (flash) revolving about fixation. On every rotational cycle (60 cycles/trial x 534 ms per cycle = 32.04 s), a sound occurred in synchrony with the same angular position of the flash. For each cycle, observers (n=10) had to report on the perceived location of the flash coincident with the perception of sound (audio-visual synchrony). The initial estimate of the location of audio-visual synchrony was slightly behind veridical audio-visual synchrony, but then over subsequent cycles, gradually moved forward in the direction of the motion. This steady drift was followed by a sharp reset back to the initially judged position. This perceptual sequence was qualitatively common across observers. When we manipulated attention to specific positions of the cycle on a trial, the location of audio-visual synchrony moved and remained there. The results show a striking parallel to the continuous report data suggesting the transitions are mediated by an attractive effect on audiovisual timing exerted by attention. Motion seems to drag spatial attention forward from the location of veridical audio-visual synchrony. As attention drifts far beyond veridical, its effect is overruled by the near-veridical sensory synchrony signals and the perceived position of audio-visual synchrony snaps back to near veridical.

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Citation

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171. On the possible relationship between orientation and choice

Harald Stoegbauer, Simone Wehling[*]*, Joydeep Bhattacharya*[*]*, Shinsuke Shimojo*

Making preferential choices is a fundamental aspect of human life. Research efforts, so far, are heavily concentrated on explaining the choice by exogenous features of the stimuli, whereas little is known about the possible role of endogenous components such as orientation responses. This latter issue is extremely relevant when the features of the possible choices are very close.

In this study, we monitored the gaze of ten observers in five two-alternative-forced-choice tasks (2AFC) where they were asked to choose a face which was more (i) attractive (ATTR), (ii) sympathetic (LIKE), (iii) unfriendly (DISL), and (iv) round (ROUN). An additional ATTR task was made with abstract pictures (ABST). The stimuli (faces or abstract) were matched according to an independent rating. Different gaze features, such as dwell times, number of fixations, likelihood of first fixation on the choice or the left stimulus, were analysed.

General properties of 2AFC: Two significant features are common to all tasks: (1) a shorter first dwell time (Friedman, $p<0.001$) and (ii). An overall longer dwell time on the chosen stimulus (paired t test, $p<0.0001$) Task differences: We found for ABST trials three features (shorter first, second last and total dwell times) that allowed their classification with a 56% accuracy, (by a chance level of 20%). Among the face tasks, only the first dwell times showed significant differences (Friedman, $p<0.003$), in which ROUN exhibits the shortest dwell and separates from ATTR, LIKE and DISL.

These results indicate an overall association, albeit weak, between orientation and choice. The next step is to investigate the directional information of this association, i.e., orientation influences choice or vice versa; although this study does not conclusively support the former, the next step must be the extension to imaging techniques.

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172. Cyclopean flash-lag illusion

Dylan Nieman, Romi Nijhawan[*], Beena Khurana[*], Shinsuke Shimojo

Possible physiological mechanisms to explain the flash-lag effect, in which subjects perceive a flashed item that is co-localized with a moving item as trailing behind the moving item, have been found within the retina of lower species, and in the motor pathways of humans. Here, we demonstrate flash-lag employing "second-order" moving and flashed stimuli, defined solely by their binocular-disparity, to circumvent any possible "early" contributions to the effect. A significant flash-lag effect was measured with cyclopean stimuli composed entirely of correlated random dot patterns. When the disparity-defined moving stimulus was replaced with a luminance-defined one, potentially engaging retinal mechanisms, the magnitude of the measured effect showed no significant change. Thus, in primates, though retinal mechanisms may contribute, flash-lag must be explained through cortical processes.

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Reference

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173. Direct instrumental conditioning of neural activity using real-time fMRI

Signe Bray, Shinsuke Shimojo, John P. O'Doherty[*]

Successful learning is often contingent on feedback. For example, it is thought that to learn movements, we use visual and proprioceptive feedback to generate appropriate neuro-motor commands. In instrumental conditioning, an animal or human learns to perform specific responses in order to obtain reward. Instrumental conditioning is often used by behavioral psychologists in order to train an animal (or human) to produce a desired behavior. Shaping involves reinforcing those behaviors that in a step-wise fashion are successively closer to the desired behavior, until the desired behavior is reached. Here, we aim to extend this traditional instrumental shaping approach in order to directly shape neural activity instead of overt behavior. To achieve this we scanned eight human subjects with real-time fMRI while they underwent an instrumental shaping procedure for imagined (and not actual) finger movement. Using real and imagined finger tapping as a functional localizer, we defined a region of interest (ROI) in motor/somatosensory areas for this group of subjects. We then rewarded subjects for producing activity in this ROI above a given threshold (by signaling receipt of one dollar per rewarded trial), only if this activity was produced following presentation of a specific discriminative stimulus. Over the course of four, 8-minute runs, we gradually increased this threshold according to an adaptive shaping procedure. Using this approach, we found a significant increase in activity in this ROI across training sessions during presentation of the stimulus signaling reward as opposed to a stimulus signaling no reward. Subject finger movement was monitored during this time, and we found no evidence of actual movement. This demonstrates that instrumental conditioning can be used to directly shape neural activity, even without the production of an overt behavioral response. This instrumental shaping technique can be distinguished from previous fMRI studies using real-time feedback in that here subjects are not provided with an explicit on-line representation of their neural activity, but rather are directly rewarded for successfully producing the desired neural responses. This approach may be relevant in the future development of therapies for stroke and other brain disorders.

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Summary: The central focus of our research is the study of learning and memory formation in freely behaving animals at the level of networks of neurons. Previous research has shown that the hippocampus is critical for the formation of long-term memories, and that this hippocampal involvement is time-limited. The current predominant conjecture is that memories are gradually established across distributed neocortical networks through the interactions between cortical and hippocampal circuits.

However, the direct experimental investigation of these interactions has been difficult since, until recently, simultaneous chronic recordings from large numbers of well-isolated single neurons were not technically feasible. These experiments became possible with the advent of the technique of chronic multi-area tetrode recordings in freely behaving rodents. Using this technique we monitor the simultaneous activity of large numbers of cortical and hippocampal cells during the acquisition and performance of memory tasks, as well as during the sleep periods preceding and following experience.

Our research efforts focus on analyzing the structure of cortico-hippocampal interactions in the different brain states and on characterizing how this structure is modulated by behavior, how it evolves throughout the learning process, and what it reflects about the intrinsic organization of memory processing at the level of networks of neurons. Our experimental work is complemented by theoretical studies of network models and the development of tools for the analysis of multi-neuronal data.

174. Dynamics of phase locking across prefrontal-hippocampal networks

Evgueniy Lubenov, Thanos Siapas

During awake behavior hippocampal activity is marked by the presence of pronounced 4-10 Hz LFP oscillations known as theta oscillations [1]. We demonstrated that a significant proportion (about 40%) of the cells in the medial prefrontal cortex of the rat fire preferentially during particular phases of the hippocampal theta rhythm [2]. Furthermore, we showed that prefrontal neurons phase lock best to theta oscillations delayed by approximately 50 ms and confirmed this hippocampo-prefrontal directionality and timing at the level of correlations between single cells. Finally, phase locking of prefrontal cells is predicted by the presence of significant correlations with hippocampal cells at positive delays up to 150 ms. This indicates that direct hippocampal input is likely to have a considerable contribution to the observed prefrontal phase locking. We are currently characterizing how these phase-locking properties evolve over time and how they are modulated by behavior.

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175. Decoupling through synchrony in recurrent networks

Evgueniy Lubenov, Thanos Siapas

Synchronization is a key property of many biological, physical, and engineering systems. The level of synchronization in distributed systems is often controlled by the strength of the interactions between individual elements. In brain circuits the connection strengths between neurons are constantly modified under the influence of spike-timing-dependent plasticity rules (STDP). However, the consequences of these local rules for the global dynamics of brain networks are not fully understood. We have shown that when recurrent networks exhibit population bursts STDP rules exert a strong decoupling force that desynchronizes activity. This decoupling force may be engaged through highly synchronous population events, known as sharp-wave bursts, which occur throughout the hippocampal formation during slow-wave sleep. We are currently analyzing the implications of these results for the transfer of information in cortico-hippocampal networks during memory formation.

176. Prefrontal-hippocampal interactions in eyeblink conditioning

Casimir Wierzynski, Thanos Siapas

Eyeblink conditioning is a form of associative learning that has been shown to engage the hippocampus across a wide range of species and parameters [1]. Moreover, in its trace form, where the conditioned and unconditioned stimuli do not overlap in time, eyeblink conditioning has been shown to require an intact hippocampus for successful acquisition [2]. This hippocampal dependence falls off with time, implying that the long-term locus of the CS-US association is extra-hippocampal. Furthermore, lesions to the medial prefrontal cortex in rats have been shown to disrupt the long-term recall of the eyeblink response, but not its acquisition [3]. Using simultaneous chronic recordings from the hippocampus and medial prefrontal cortex, we are characterizing the relationships between the activity patterns in these brain areas during the acquisition of the CS-US association, with the eventual goal of understanding how this association is represented across the prefrontal-hippocampal networks.

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177. Spatial firing properties of cells across the DG, CA3, and CA1 hippocampal subfields

Ming Gu, Thanos Siapas

The hippocampus consists of several subfields with different connectivity and electrophysiological properties. The characteristic organization of each region has inspired many models of the computational roles of each subfield in memory formation. However, experimental support for the different models has been limited [1]. Using simultaneous recordings from the DG, CA3, and CA1 hippocampal subfields, we are interested in characterizing how activity patterns get transformed across these subfields during the acquisition and performance of spatial learning tasks. Our current efforts focus on characterizing and comparing the spatial firing properties of DG, CA3, and CA1 cells, and quantifying how these properties change as a function of learning.

Reference

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178. Reward learning and VTA-hippocampal interactions

Thanos Siapas

Many lines of evidence suggest that the ventral tegmental area (VTA) interacts with the hippocampus to modulate the entry of information into long-term memory [1]. The VTA-hippocampal loop has been hypothesized to be important for the detection of novelty and signaling of the behavioral relevance of stimuli in the environment. However, the organization of VTA-hippocampal interactions remains unknown, as simultaneous recordings from these areas have not been reported in the literature. Leveraging our experience with chronic multi-area tetrode recordings, we are examining the simultaneous response of multiple dopaminergic cells in the VTA and pyramidal cells in hippocampus during exposure to novel stimuli as well as during the acquisition of instrumental conditioning paradigms.

Reference

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179. Wireless large-scale recordings of neuronal activity in freely behaving animals

Evgueniy Lubenov, Thanos Siapas

In collaboration with Alan Litke of the University of California, Santa Cruz, and Markus Meister of Harvard University, we are developing a wireless system that would allow recording neuronal activity from freely-behaving animals. The system will acquire signals from 64 channels, amplify and filter the waveform from each channel, and transmit the data with a broadband radio transmitter to a remote receiver. This technique would allow examining neuronal network activity while animals explore much larger spatial extends than previously possible, and may enable probing neural function in natural behaviors, such as flying, that were difficult to study in the past.

Pubication

Siapas, A.G., Lubenov, E.V. and Wilson, M.A. (2005) Prefrontal phase locking to hippocampal theta oscillations. *NEURON* **46**(1):141-151.

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Summary: Our group studies the molecular mechanisms of axon guidance and synaptogenesis. The fruit fly *Drosophila melanogaster* is our primary experimental system. In the embryo, we examine signaling systems involved in motor and CNS axon guidance. In the larva, we study synaptogenesis and synaptic plasticity in the neuromuscular system. Our approaches combine genetics, molecular biology, electrophysiology, biochemistry, and cell biology.

Motor axon guidance. The *Drosophila* motor axon network has provided one of the best systems in which to study growth cone pathfinding mechanisms. The network is simple: 32 motoneurons innervate 30 body wall muscle fibers in each abdominal segment. Each motoneuron axon is targeted to a specific muscle fiber, and very few projection errors are made during normal development. Thus, the motor axon network is a genetically hard-wired map, and is an ideal system in which to study how genes control the formation of specific synaptic connections. In much of our work, we have focused on the roles of tyrosine phosphorylation in regulating motor axon guidance decisions. We are now also conducting screens to determine the mechanisms by which cell surface proteins label specific muscle fibers for recognition by motor axon growth cones.

Neural receptor tyrosine phosphatases. In the 1990's, we showed that receptor-linked protein tyrosine phosphatases (RPTPs) are selectively expressed on CNS axons and growth cones in the *Drosophila* embryo, and that these RPTPs regulate motor and CNS axon guidance during embryonic development. RPTPs directly couple cell recognition *via* their extracellular domains to control of tyrosine phosphorylation *via* their cytoplasmic enzymatic domains. The extracellular regions of the fly RPTPs all contain immunoglobulin-like (Ig) and/or fibronectin type III (FN3) domains, which are usually involved in recognition of cell-surface or extracellular matrix ligands. Their cytoplasmic regions contain either one or two PTP enzymatic domains. The fly genome encodes six RPTPs (LAR, DPTP10D, DPTP69D, DPTP99A, DPTP52F, DPTP4E), and we have generated or obtained mutations in all six of the genes encoding these proteins (Jeon abstract).

We have now performed a detailed characterization of the genetic interactions among all six RPTPs. We find that each growth cone guidance decision in the neuromuscular system has a requirement for a unique subset of RPTPs; thus, in a sense, there is an "RPTP code" for each decision. In some cases, the RPTPs work together, so that defects are only observed when two or more are removed. In other cases, however, phenotypes produced by removal of one RPTP are suppressed when a second RPTP is also absent. Our results provide evidence for three types of relationships among the RPTPs: partial redundancy; collaboration; and competition. Our major efforts in this area are now directed toward understanding these relationships at the biochemical level, through definition of upstream (ligands) and downstream (substrates) components of RPTP signaling pathways (see below).

Searching for RPTP substrates. It is difficult to identify PTP substrates biochemically because PTPs usually do not display strong specificity *in vitro*. To find substrates, we performed yeast two-hybrid screens with 'substrate-trap' mutant versions of DPTP10D, DPTP69D, DPTP52F, and DPTP99A. These 'trap' proteins form stable complexes with tyrosine-phosphorylated substrates because they bind normally but cannot catalyze dephosphorylation. We introduced a constitutively activated chicken Src tyrosine kinase into yeast together with the PTP trap constructs and the cDNA library, in the hope that it would phosphorylate relevant substrate fusion proteins made from cDNA library plasmids. We identified several classes of clones whose interactions with the substrate-trap RPTPs are dependent on coexpression of the tyrosine kinase, suggesting that they may be substrates. These include a cell-surface receptor and some intracellular signaling proteins. We are currently examining whether these proteins interact with the RPTPs *in vivo* and if they are required for RPTP signaling (Bugga abstract).

Identification of RPTP ligands. The ligands recognized by RPTPs *in vivo* have not been identified in any system. In order to understand how RPTPs regulate axon guidance, it is essential to know when and where they engage ligands, and how ligand binding affects enzymatic activity and/or localization.

Our current approach to identifying ligands is based on our observation that fusion proteins in which the extracellular domains of RPTPs are joined to human placental alkaline phosphatase (AP) can be used to stain live *Drosophila* embryos. Each of the six fusion proteins (LAR-AP, DPTP69D-AP, DPTP10D-AP, DPTP99A-AP, DPTP4E-AP, DPTP52F-AP) binds in a specific manner. Each fusion protein stains a subset of CNS axons and also binds to other cell types in the periphery. To identify the genes encoding the RPTP ligands, we are screening deficiency mutations that remove specific portions of the

genome. We began by screening the Bloomington 'deficiency (Df) kit' of 266 fly lines. Each Df line was crossed to GFP balancers so that Df/Df embryos could be identified, and we then stained these embryos with each of four fusion proteins (LAR-HS2-AP, 69D-AP, 10D-AP, 99A-AP). Since each Df lacks a specific region of the genome, if homozygous Df embryos don't stain with a fusion protein, this indicates that this genomic region contains a gene required for ligand expression. Overlapping Dfs and point mutants can then be screened in order to identify the relevant gene within the Df (also see Wright abstract).

Using this screen, we have already found a Df that contains a gene encoding a ligand that binds to LAR-AP, and have identified this ligand as Syndecan (Sdc). This work was recently published*. The heparan sulfate proteoglycan Syndecan is an *in vivo* ligand for the *Drosophila* LAR receptor tyrosine phosphatase. Sdc is a heparan sulfate proteoglycan (HSPG). Our results show that LAR binds to the glycosaminoglycan side chains of Sdc with nanomolar affinity, and that Sdc is required for DLAR-mediated axon guidance. We can generate motor axon guidance errors by overexpressing LAR on neurons, and find that the same errors are generated by ectopically expressing Sdc on muscles. This Sdc gain-of-function (GOF) phenotype is suppressed by loss-of-function (LOF) mutations in the *Lar* gene, indicating that LAR is epistatic to (downstream of) Sdc. This result shows that muscle Sdc can function as a ligand for LAR on neuronal growth cones, and suggests that binding to Sdc increases LAR's signaling activity.

We have continued the Df screen, and have identified four regions required for 99A-AP staining, and three regions required for HS2-AP staining. We are now screening overlapping Dfs and point mutations to try to find the responsible genes.

Reference

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Targeting of motor axons to specific muscle fibers. Despite the advances in characterizing molecules that regulate motor axon pathfinding, we still understand little about how specific muscle fibers are recognized as targets for synapse formation by these axons. Many mutations affect pathfinding decisions, leading to aberrant wiring of the neuromuscular system, but no single LOF mutations are known that block recognition of specific muscle targets. These results are most easily explained by invoking genetic redundancy in target labeling. If each muscle fiber were defined by a combination of several cell-surface labels, removing one of the labels might not have a major effect on targeting of axons to that fiber. This would explain why targeting molecules have not been identified in conventional loss-of-function (LOF) genetic screens.

Studies of gain-of-function (GOF) phenotypes by other groups are consistent with the redundancy hypothesis. For example, the homophilic cell adhesion molecule Fasciclin III (Fas III) is expressed on only two muscle fibers, 6 and 7, and on the growth cone of the RP3 neuron that innervates these two fibers. Fas III appears to be a functional target label, because when it is ectopically expressed on other muscle fibers near 6 and 7, the RP3 neuron makes abnormal synapses on these Fas III-expressing fibers. However, when Fas III is removed by a LOF mutation, there is no effect on targeting of RP3 to 6 and 7. These results imply that Fas III can be used for muscle targeting, but that targeting of 6 and 7 can still proceed in its absence, presumably because these fibers are also labeled by other surface molecules that can be detected by the RP3 growth cone when Fas III is not present.

These findings suggest that cell-surface proteins that label specific targets in the motor axon system might be identifiable by a GOF genetic screen in which candidate labels are ectopically expressed on all muscle fibers. If these proteins are functional labels, their misexpression might produce alterations in target recognition, as observed in the Fas III experiments described above. By identifying all the genes encoded in the *Drosophila* genome that can confer GOF phenotypes in which targeting of specific muscle fibers is altered, we will acquire the tools to understand the mechanisms involved in target recognition in this system. This type of screen should allow us to overcome the redundancy problem. For example, suppose one could identify three different cell-surface proteins that are normally expressed on a specific muscle fiber, but whose misexpression on other muscle fibers produces targeting errors. One might then predict that removing all three of these proteins by making a triple LOF mutant (through conventional or RNAi techniques) would now prevent targeting of this muscle fiber. Through these kinds of experiments, we could begin to understand the combinatorial code for muscle targeting. Insights into the motor axon targeting code would be likely to facilitate an understanding of targeting in other neuronal systems (e.g., the antennal lobe, optic lobe, and mushroom body), since candidate target labels are usually expressed by a variety of neuronal and non-neuronal cell types.

To conduct this GOF screen, we first created a database of all cell-surface and secreted (CSS) proteins in *Drosophila* that are likely to be involved in specific cell-cell interactions. The database was generated by defining all fly genes encoding proteins that contain domains known to be present in CSS proteins in other eukaryotes. It currently contains 1005 genes.

To drive expression of these genes in muscles, we used the 'EP' system, in which a P element containing a block of UAS sequences that are responsive to the yeast transcription factor GAL4 is jumped around the genome. Like other P elements, EPs usually land upstream of genes. If a line bearing an EP upstream of a gene is crossed to a 'driver' line expressing GAL4 in all muscle fibers, the gene will now be expressed at high levels in muscles in the resulting progeny embryos and larvae. To find EP-like elements upstream of the CSS genes, we searched through about 40,000 different insertions, including the original EP

set generated by Pernille Rorth[1], the EY insertions lines generated in the Bellen lab[2], the GS lines developed in Japan, and the GE lines developed by GenExel, Inc. We were able to identify insertions that can confer expression of about 400 of the 1005 CSS genes in our database, representing members of all CSS protein families.

To screen for genes encoding potential targeting molecules, we are crossing each of these insertions to a muscle GAL4 driver and visualizing motor axons and neuromuscular junction synapses in the resulting F1 progeny larvae by immunostaining. We have already identified a quite a few genes that cause specific mistargeting or synaptic morphology phenotypes when they are expressed on muscles (Cording abstract).

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Genes controlling synaptogenesis in the larval neuromuscular system. Motor growth cones reach their muscle targets during late embryogenesis and then mature into presynaptic terminals that are functional by the time of hatching. The pattern of Type I neuromuscular junction (NMJ) synapses in the larva is simple and highly stereotyped, with boutons restricted to specific locations on each muscle fiber. These synapses continue to expand and change as the larva grows, because their strengths must be matched to the sizes of the muscle fibers they drive. This growth represents a form of synaptic plasticity, because it is controlled by feedback from the muscle to the neuron. Studies of NMJ synapses in flies are relevant to an understanding of synaptic plasticity in the mammalian brain, because the fly NMJ is a glutamatergic synapse, organized into boutons, that uses ionotropic glutamate receptors homologous to vertebrate AMPA receptors.

Control of synaptic local translation. Our recent work on synapses has focused on control of synaptic protein translation. Local translation at synapses has been studied in *Aplysia*, mammalian, and arthropod systems. It has attracted interest because it is a mechanism that allows neurons to separately adjust the strengths of individual synapses.

To identify genes involved in synaptogenesis in larvae, including those that regulate local translation, we devised and executed a GOF screen of live third instar larvae (Kraut *et al*., 2001; PDF available on website). In the screen, we identified *pumilio* (*pum*), which encodes an RNA-binding protein that shuts down translation of specific mRNAs by binding to their 3' untranslated regions. Translational repression by Pum controls posterior patterning during embryonic development. In a recent *Neuron* paper (PDF available on website), we showed that Pum is an important mediator of synaptic growth and plasticity at the NMJ. Pum is localized to the postsynaptic side of the NMJ in third instar larvae, and is also expressed in larval neurons. Neuronal Pum regulates synaptic growth. In its absence, NMJ boutons are larger and fewer in number, while Pum overexpression increases bouton number and decreases bouton size. Postsynaptic Pum negatively regulates expression of the essential translation factor eIF-4E (the cap-binding protein) at the NMJ, and Pum binds selectively to the 3'UTR of *eIF 4E* mRNA. These data suggest that Pum is a direct regulator of local eIF-4E translation, and that eIF-4E (which is normally limiting for translation) in turn switches on translation of other synaptic mRNAs. These mRNAs probably include that encoding the GluRIIa glutamate receptor, which is also upregulated in *pum* mutants. These results, together with genetic epistasis studies, suggest that postsynaptic Pum modulates synaptic function *via* direct control of local synaptic translation.

We are now examining a number of other RNA-binding proteins that may regulate postsynaptic translation at the NMJ (Menon abstract). These include FMRP (Fragile X mental retardation protein ortholog), Orb (CPEB ortholog), Nanos (Pum's partner during early development), and Cup (a protein that regulates eIF-4E activity). We are also studying whether controlled aggregation of Pum regulates its ability to repress translation. This is being studied both *in vivo* and *in vitro* (Silverman abstract).

180. Identifying cell-surface proteins involved in targeting of motor axons to specific muscle fibers

Amy Cording, Mitsuhiko Kurusu[*]*, Kai Zinn*

We used computer analysis of sequence databases to define a set of ~1000 genes in *Drosophila* that encode cell-surface or secreted (CSS) proteins that could be involved in cell recognition events. We obtained 'EP'-like P element insertion lines, in which a UAS element is located upstream of the gene and can be used to drive its expression, for >400 of these genes. Each of these was crossed to a pan-muscle GAL4 driver, 24B, and neuromuscular junctions (NMJ) and motor axons examined in the resulting larvae, which are expected to express the relevant CSS protein at high levels in all muscles. We found many genes for which pan-muscle (postsynaptic) expression produces dramatic alterations of the presynaptic terminal. These may reveal new pathways for feedback from postsynaptic to presynaptic cells. Our major focus in the past year, however, has been on a smaller group of genes for which pan-muscle expression causes mistargeting of axons that normally innervate muscle 12. We observed that targeting of ISNb axons, including RP5 and V, to muscle 12 was most affected by misexpression, and so focused on this decision. In recent work, we crossed the candidate mistargeting lines to other muscle and neuronal drivers to verify the phenotypes and determine the timing of expression required to generate them. We then performed *in situ* hybridization experiments to prove that the candidate gene was in fact, driven by GAL4, and to examine the endogenous expression pattern of the gene. We have completed this analysis for >10 genes already. We will continue these experiments until we have obtained a complete dataset for each of the mistargeting genes. At the same time, we are generating or ordering loss-of-function mutations for the

genes of most interest, and examining whether these cause motor axon phenotypes.

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181. Identification of substrates for RPTPs

Lakshmi Bugga

To isolate *Drosophila* neuronal RPTPs-10D, 69D, 99A and 52F in a complex with their target substrates, we are using yeast two-hybrid system. To attempt to achieve stable binding of the RPTPs to a tyrosine phosphorylated substrate, we have used 'substrate-trap' mutants of the RPTPs, which can bind to substrates but do not catalyze dephosphorylation, instead remaining bound to substrate in a stable complex. We constructed plasmids encoding GAL4 DBD/RPTP bait proteins and introduced them into yeast together with fly cDNA libraries encoding GAL4AD-cDNA fusion proteins. We also introduced a plasmid containing a constitutively active form of chicken c-Src, driven by a constitutive yeast promoter. Positive interactions are detected by selecting on plates lacking the auxotrophic marker and screening for reporter expression and src dependence.

Our yeast two-hybrid screen with *Drosophila* neuronal RPTPs-10D and 52F resulted in four potential substrates based on Src dependence. Of the four genes, two are known genes - Tartan (a cell adhesion molecule expressed in embryonic CNS and PNS), Xmas-1 (a RNA-binding protein that is involved in spermatogenesis, oogenesis and embryogenesis). Of the two unknown genes, one is rich in proline residues and also has proline motifs, which are known to bind to SH3 domains. RNA *in situs* with this gene showed expression in the embryonic CNS.

We are currently testing these interactions *in vitro* by transient transfection experiments with *Drosophila* S2 cell lines and finding out the expression of rest of the unknown genes by RNA *in situs*, and looking for double mutant phenotypes with DPTP and tartan mutant flies. Our preliminary experiments with tartan mutant flies showed same neuronal defects as 52F. Our biochemical experiments with full-length tartan showed association with substrate-trap 52f, and this association decreased/disappeared with the wild-type 52F, suggesting that tartan is a substrate for 52f. We are trying to map the area of the tartan gene that specifically interacts with 52F by mutating either single or combination of tyrosine residues.

182. Translational control mechanisms at the larval neuromuscular junction

Kaushiki P. Menon

Pumilio is a translational repressor that localizes postsynaptically at larval neuromuscular junctions (NMJs). It is required both pre- and post-synaptically in the nervous system. In neurons, it is required for synaptic growth. Postsynaptically, it represses accumulation of the cap-binding protein, Eukaryotic Initiation Factor eIF4E at the NMJ. eIF4E accumulation has been shown to control both morphological and functional properties of the larval NMJ. Pumilio binds specifically to the 3' untranslated region of eIF4E mRNA and thereby presumably controlling eIF4E accumulation and indirectly controlling translation of glutamate receptor, GluRIIA mRNA at the NMJ.

Pumilio interacts with Nanos and Brat proteins to repress translation of target *hunchback* mRNA in early embryogenesis in *Drosophila*. We are therefore, exploring the role of Nanos in synaptogenesis. We have found that Nanos shows similar expression pattern as Pumilio, both at the NMJ and in the CNS. Nanos when overexpressed in neurons shows a structural synaptic phenotype distinct from neuronal overexpression of Pumilio. *Nanos* mutants show defects in electrophysiologically and morphologically properties. We are currently examining *nanos* rescue lines to see if this phenotype is restored to normal. Since none of the existing mutants are complete nulls, we are also making RNAi lines for *nanos*. We will be expressing the RNAi lines separately in the somatic muscles and in the neurons to dissect the role of Nanos in these two tissues.

Recently, several reports have shown that Cup, a germline protein, interacts with eIF4E protein and represses its function in the developing embryo. We have found that Cup protein shows similar localization patterns as Pumilio and Nanos at the larval NMJ. Cup mutants also have electrophysiological and morphological defects at the NMJ. We are currently determining how Pumilio, Nanos and Cup interact with each other to regulate synaptic function at the NMJ.

183. The *in vitro* aggregation of an NQ-rich region of *pumilio*

Ed Silverman

We are interested in whether the NQ (asparagine/glutamine)-rich region of *pumilio* has the capability to aggregate and, if so, whether this aggregation can alter the function of *pumilio*. Our lab has shown that a Q/N-rich region of *pumilio* can aggregate in yeast and studies are currently under way investigating potential *in vivo* effects in flies (see Salazar abstract). Another way we are addressing this is by analyzing the NQ-rich region of *pumilio in vitro*. Using a canonical NQ-rich region of yeast protein Sup35 known to aggregate in a prion-like manner, NM, as a positive control, and the highly charged M region of Sup35 as a negative control, we purified the first NQ-rich region of pum (NQ1) and NQ1 lacking glutamine-rich stretches (NQ-Q) and allowed these proteins to spontaneously convert with mild agitation into an aggregated state.

Several assays can be used to determine whether a protein aggregates into a stable, amyloid-like form. First, we looked at solubility in 2% SDS. A protein capable of forming amyloid will be insoluble in SDS without boiling. After conversion, NQ1 and NM are insoluble in SDS without boiling; whereas, M remains soluble and NQ-Q is mostly soluble. This result indicates that NQ1 is capable of forming amyloid. To confirm this we stained the converted fibers of NQ1 and NM with Congo Red, and looked for birefringence, which is used to diagnose amyloid in disease and *in vitro*. Both the NQ1 and NM

proteins formed small macroscopic fibers that bound Congo Red, and exhibited birefringence in cross-polarized light. The conversion reactions of NQ1 and NM are recognized by an antibody that is specific against amyloid oligomers that are precursors to amyloid formation. We are currently examining the kinetics of amyloid formation more closely, looking at seeding and temperature effects, and plan to test a yeast strain carrying a chimeric gene where NQ1 replaces the NM region of Sup35 for the ability of the *in vitro* fibers to be infectious. We also plan to examine the fibers under EM to see small fragments and determine whether they have properties typical of an amyloid. We will also test other regions of *pumilio* and accessory domains (non NQ) in NQ1 to better understand the conversion and aggregation events.

184. Translational regulation of PABP at the *Drosophila* neuromuscular junction

Ed Silverman, Kaushiki Menon

Poly(A)-binding protein (PABP) binds to the poly(A) tail present on most eukaryotic mRNAs. Cytoplasmic PABP is important for translational initiation, binding to the poly(A) tail and interacting with eIF4G to form a loop linking the 5' and 3' ends of the mRNA. This inhibits decapping and promotes translation. We are interested in studying the translational regulation of the PABP because there is evidence implicating the importance of PABP regulation for synaptic plasticity. Alteration of PABP expression leads to an increase in postsynaptic aggregates and changes synaptic morphology. Both PABP and eIF4E can form aggregates in response to activity that appear as puncta, which can be controlled by temperature and food consistency. The PABP aggregates appear to colocalize with eIF4E [Sigrist *et al*. (2000) *Nature* **405**:1062-1065]. Our lab has shown that eIF4E is directly regulated postsynaptically by Pumilio (Pum) binding to a specific region of the eIF4E 3'UTR; this regulation affects local translation and synaptic plasticity by controlling expression of the GluRIIa receptor [Menon *et al.* (2004) *Neuron* **44**:663–676]. Interestingly, it has been shown that eIF4E aggregates only form in response to altered PABP levels in wild-type flies when movement is induced [Sigrist *et al.* (2003) *J. Neurosci.* **23**(16):6546–6556]; whereas, in *pum* mutants, eIF4E aggregates even before induction of movement. This suggests a potential role for Pum in the regulation of PABP. PABP levels appear to be upregulated throughout the muscle in *pum* mutants; we are currently in the process of quantifying PABP puncta in *pum* mutant larvae.

PABP has a long 3'UTR of approximately 1500 nucleotides in larval muscle preparations, so in order to determine which areas were important, we aligned the 3'UTR sequences with the homologous sequences from six other *Drosophila* species and identified eight distinct areas of enhanced conservation. We intend to fuse the 3'UTR of PABP to GFP and create mutants encompassing these conserved elements in order to elucidate how PABP expression is regulated. One of the conserved elements (CE), CE1, is a putative target site for the mir-305, a microRNA. If removing CE1 deregulates the GFP-*pabp3'UTR* chimera, we will investigate the role of RISC and other members of the miRNA processing pathway for effects on PABP. Two other conserved elements have a resemblance to a Pum binding site. If changing any of the other conserved elements deregulates PABP we will examine the role of Pum or other translational regulators for physical interaction with the *pabp 3'UTR* and genetic interaction with *Pabp*. We will then attempt to determine the contribution of each identified regulator of PABP to synaptic plasticity.

185. A deficiency screen for axon guidance defects in *Drosophila melanogaster*

Ashley Wright

Only one systematic screen for axon guidance and muscle targeting defects has been carried out in *Drosophila* embryos*. In that study, heavily mutagenized second chromosomes (40% of the genome) were screened for defects by staining with an antibody that labels motor neurons in whole-mount embryos. By their estimate, only 50% of the motor projections in the animal can be visualized by this method. The authors found ten genes and estimate that there may be upwards of 20 genes, which when mutated one at a time can lead to axon guidance or targeting defects. We are currently carrying out a screen using a subset of the *Drosophila* deficiency kit which have wild-type or nearly wild-type central nervous system development and we hope to identify more genes or gene families which are involved in these processes. In order to visualize all motor projections in the embryos, we are carrying out live dissections on each line that opens up the body wall so that all motor projections are visible. These dissected embryos are then stained with the same antibody used by Vanvactor *et al*. (1993), and scored for defects.

Reference

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Publication

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Summary: In the past year, significant advances have been made in several projects that have been pursued in our laboratory in the last few years, as well as in new directions that our research has taken. Thus, an analysis of the main mtDNA control region in fibroblasts of subjects older than 65 years has revealed several mutations either at a site adjacent to the replication origin at position 151 (i.e., a C150T or a T152C transition), or at a site close to the replication origin at position 191 (i.e., a T195C transition), or at a site corresponding to the 414 promoter for the RNA primer of heavy stand synthesis (in particular, T414G transversion). Most interestingly, while the C150T transition in Italian centenarians was associated with a change in the mtDNA replication origin at position 151 to position 149, the aging-related heteroplasmic T152C transition in Ashkenazi Jews was not accompanied by any change in such replication origin.

A very significant finding was the occurrence in fibroblasts and immortalized lymphocytes of a novel interaction between human mtDNA and a TCA cycle enzyme. This enzyme turned out to be the mitochondrial malate dehydrogenase. Overexpression of this enzyme in HeLa cells resulted in an increase in mtDNA copy number and in an activation of the mtDNA origins.

186. Investigation of a novel interaction between mtDNA and a TCA cycle enzyme in human mitochondria

Jaehyoung Cho

Mitochondria have their own genome, and cooperate with the nuclear genome in regulating respiration, energy metabolism, and, more surprisingly, aging and longevity (1, 2). Recent papers reported that mitochondrial metabolic enzymes bind to mtDNA and have a direct function for mtDNA biogenesis (3, 4). The D-loop region of human mitochondrial DNA (mtDNA) is essential to control replication and transcription, and this control region also undergoes some mutations which occur in aging-dependent manner (1, 2).

To investigate the molecular basis of the aging-related mtDNA mutagenesis and the selection process involved, we planned to identify possible protein-DNA interactions in this region. During the replication process, an early intermediate mtDNA form is a long, triple-stranded D-loop. The stability of the single-stranded region of the D-loop may have an important role in the regulation of mtDNA copy number. Thus, we searched for possible mitochondrial protein-mtDNA interactions in both the single-stranded DNAs (heavy and light strands) of this region. At first, we focused on the DLP4 region of the D-loop because this segment of mtDNA is the locus of several important point mutations related to human aging, in particular, the C150T, T152C and A189G mutations (2). In addition, this region harbors major replication origins and the CSB1 (conserved sequence block 1). Very interestingly, only the light strand of the DLP4 segment showed binding activity to several proteins in DNA mobility shift assays. We also tested the specific DNA-protein interactions in small subregions of DLP4 and found that each subregion has a different profile of DNA-protein complex. The results showed that the first three subregions have stronger protein binding activity than the remaining regions.

We purified the proteins by single-stranded DNA (ssDNA) affinity chromatography, and found that these proteins have molecular weights of 70, 55, 35, 28 and 15 kDa. Among them, the 35 kDa protein shows specific binding to a previously known origin of heavy-strand mtDNA replication (OH2). This OH2 region is the site of a very interesting aging-dependent mutation (C150T). Interestingly, the 35 kDa protein interacts preferentially with the mutant sequence rather than with the wild-type sequence. Since the 5'-end analysis of nascent heavy strands consistently revealed a new replication origin at position 149, substituting for that at 151, only in C150T mutation-carrying samples of fibroblasts or immortalized lymphocytes (2), the 35 kDa protein (p35) may have an important role for the selection of a remodeled replication origin. We characterized the molecular nature of this protein by LC/MS/MS (liquid chromatography and tandem mass spectrometry). The results showed that this protein is a critical enzyme of the TCA cycle; i.e., the mitochondrial malate dehydrogenase. We confirmed that the commercially available p35 purified from pig heart can also bind specifically to the DLP4-L2 subregion, which includes OH2, *in vitro*, and that the antibody against this protein can make a super-shifted band in a DNA mobility shift assay. We also verified the specificity of p35-OH2 interaction by DNase I footprinting analysis. The ssDNA interaction inhibited the enzyme activity of p35 in a sequence specific manner. Very interestingly, this interaction was inhibited by NADH in a dose-dependent manner. To investigate the role of this interaction in mtDNA biogenesis, we overexpressed human p35 in HeLa cell, and then examined the content and the replication of mtDNA. We confirmed the overexpression of p35 by an immunoblot assay and tested also the contents of other mitochondrial proteins important for mtDNA metabolism. We found that the mtDNA copy number increases in p35-overexpressing HeLa cells, depending on the expression level of p35. We also tested the usage of replication origins during overexpression of p35, and the

results showed that the replication origins of mtDNA were also turned on in the same manner. In a previous report, this laboratory showed that human mtDNA makes a specific DNA-protein complex in the D-loop region forming a novel knob structure (5). Therefore, we purified the mtDNA-protein complex from highly purified mitochondria to test if this knob structure contains p35 or not. The results showed that the purified knob structure has very similar binding protein profiles to that observed in a DNA affinity chromatography using DLP4-L2. The same size of 35 kDa protein was also found as a component of the mtDNA-protein complex, and characterized as p35 by immunoblot analysis.

Our data strongly support the conclusion that two important factors, i.e., energy metabolism and mtDNA function, regulate the aging process during the lifespan.

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187. Two-color-single-base extension genotyping (2C-SBEG) of aging-dependent human mitochondrial DNA polymorphisms

Jaehyoung Cho

The loss of integrity of human mitochondrial DNA (mtDNA) is closely related to human metabolic disorders and aging. In the past few years, we have reported the occurrence of an aging-dependent large accumulation of point mutations in the mtDNA main control region of leukocytes in the majority of individuals above a certain age. To understand the role of these point mutations in human aging, a fast and accurate genotyping of a large number of samples is essential. The presently available methods, such as DGGE (denaturing gradient gel electrophoresis), SSCP (single-strand conformation polymorphism) and differential primer extension technique, require several time-consuming steps including radiolabeling, and are not applicable for large-scale analysis. Recently, another efficient method using quantitative PCR has been developed for large-scale genotyping, but this method is too expensive. Therefore, we planned to develop an efficient way to analyze polymorphisms of human mtDNA on a large scale.

In the differential primer extension technique, a radiolabeled primer targeted to a specific locus is extended by Vent DNA polymerase, and the extension products are differentially terminated by incorporation of a dideoxynucleotide at the 3' end. The extended primers carrying wild-type or mutated mtDNA sequence have different length, and can be analyzed by polyacrylamide-urea gel electrophoresis. Usually, the whole procedure requires a whole week, and the quantification of the polymorphism is not possible in some cases. We have modified the original procedure by using different fluorescent dye-labeled ddNTP mixtures. In this new method, a single base is incorporated at the 3' end of an unlabeled primer by using two different fluorescent dye-labeled ddNTPs (for example, green Cy3-ddCTP and red Cy5-ddTTP). The extended primers are blotted on a Zeta-probe nylon membrane using a 96-well dot blotting system, and the membrane is washed twice with buffer to remove un-incorporated fluorescent ddNTPs. The genotype of the each sample is analyzed by the color-difference detected by the Molecular Imager FX Pro Plus system (Biorad). This system is very sensitive and can detect 0.2 fmol of Cy3 or Cy5 end-labeled DNA using laser light. The quantification of polymorphisms is quite efficient and easy. The quantity and ratio of wild-type and mutant genomes can be calculated from the color difference using standard mixtures containing various known amounts of wild-type and mutant DNA. The whole procedure of this new technique just needs a single day even for a large-scale analysis, and is still economic. We named this new technique as two-color single-base extension genotyping (2C-SBEG).

By using the method described above, we detected and confirmed the occurrence of a C150T heteroplasmy as low as 20% in a human total cell (leukocyte) DNA sample. Usually, a conventional sequencing procedure cannot detect a heteroplasmic genotype under 30%. Therefore, our method may provide a significant improvement in the analysis of the various ranges of heteroplasmy in human mtDNA.

188. Aging-related mtDNA mutations in Ashkenazi Jews

Nahoko Iwata, Jin Zhang, Jaehyoung Cho, Anne Chomyn

Our previous observation that an mtDNA homoplasmic C150T transition adjacent to the heavy-strand replication origin at position 151 is greatly increased in frequency in Italian centenarians, as compared to the rest of the population, has prompted us to analyze a genetically distinct population. Thus, in collaboration with Gil Atzmon*, Suzanne Leanza*, Robert D. Burk*, and Nir Barzilai*, we have analyzed leukocyte mtDNA from three groups of an Ashkenazi Jew population, namely, a large number of female probands (95-108 years-old), their mixed-gender offspring and mixed-gender control subjects. This analysis revealed a very low incidence of the C150T transition and, by contrast, the fairly high frequency of a homoplasmic T152C transition and of a homoplasmic T195C transition in all three groups of subjects. Furthermore, most significantly, an aging-related increase in incidence of the heteroplasmic T152C transition, presumably resulting from somatic events, was

demonstrated in the Ashkenazi Jews. In these, the T152C transition was not associated with a change in the replication origin at position 151, unlike the C150T transition in the Italian centenarians.

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189. Association between the aging-dependent T414G mutation in human mtDNA and a phenotype showing improvement of mitochondrial respiration

Paola Sgobbo, Anne Chomyn

The mtDNA T414G transversion in human skin fibroblasts shows an aging-related accumulation occurring in subjects above 65 years old (1). The position of this mutation is at a critical control site for mtDNA replication, in particular, within the promoter for the RNA primer of H-strand replication. Therefore, last year, we started to investigate the role that the mutation could play, and the reason for its increase in incidence in old individuals. Our studies were focused especially on a possible association of the mutation with a particular phenotype. In particular, we studied some biochemical aspects of transformant cells in which the nuclear background is that of mtDNA-less 143B ρ^o cells, while the mtDNA background is that of mutation-carrying fibroblasts.

As previously described, we analyzed transformant cell lines carrying mitochondria that contain mtDNA either 100% mutant or 100% wild type at the 414 position. In our previous experiments we had observed that one of the non-mutant cell lines had a higher doubling time as compared to a mutant one. Subsequently, the measurement of rate of respiration, either endogenous or substrate-driven respiration, showed higher values in mutant cells than in control cells. These findings seemed to indicate, that, as a result of the mutation, there was a significant improvement in the function of the mitochondrial respiratory complexes. In order to confirm these results, we analyzed other pairs of transformant cells derived from different fibroblast donors. Our preliminary experiments have corroborated our hypothesis about a possible energetic improvement associated with the mutation. We are also sequencing the entire mitochondrial genome in these transformant cell lines in order to investigate the possible presence of multiple mutations co-segregating with the T414G, which could influence the phenotype. Further investigations will concentrate on the analysis of other pairs of transformant cells in order to validate the association of a defined phenotype with the T414G mutation.

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190. Effects of C150T mutation of mtDNA D-loop in human cybrid cells

Ai Chen, Anne Chomyn

Mitochondrial DNA (mtDNA) is highly polymorphic, and its variations in humans may contribute to individual differences in function. Recently, our laboratory has found a strikingly higher frequency of a C150T mutation in mtDNA from centenarians and twins, and has also demonstrated that the mutation causes a remodeling of the mtDNA 151 replication origin in human leukocytes and fibroblasts (Zhang *et al.,* 2003). To further understand the mechanism of the C150T mutation located in the mtDNA D-loop region, we have investigated transformants obtained by transferring human fibroblast mtDNA carrying the C150T mutation into human mtDNA–less cells (ρ^0 cells) derived from an osteosarcoma cell line. Some of the transformants used in our studies were constructed earlier (N. Raule, Biology Annual Reports, 2004, No. 181). In particular, we chose for analysis six non-mutant transmitochondrial clones derived from one fibroblast strain and six homoplasmic C150T mutation-carrying transmitochondrial clones derived from another fibroblast strain.

A biochemical characterization of these non-mutant or mutant transformants was carried out by measuring in intact cells their doubling times and their rates of total O_2 consumption, coupled or dinotrophenol (DNP)-uncoupled, or of ascorbate/N,N,N,N-tetramethyl-p-phenylenediamine (TMPD)-driven respiration. TMPD-driven respiration reflects the activity of cytochrome *c* oxidase (COX). Compared to the average levels measured in the non-mutant transformants analyzed, a reduction in doubling times, and an increase in total O_2 consumption rate, in the ratio of DNP-uncoupled to coupled endogenous respiration rates, and in the TMPD-driven respiration rate were observed in the C150T mutation-carrying transformants. We also checked the membrane potential of the cells and found that it tended to parallel the endogenous respiration rate. In addition, when we checked the reactive oxygen species (ROS) production rate in both non-mutant and C150T transformants by using of CM-H_2DCFDA as a fluorescence probe, a significantly decreased ROS production was observed in the C150T mutant transformants. We also measured the manganese superoxide dismutase (MnSOD) protein level in C150T mutant and wild-type cells, and observed that the mutant cells had higher levels of this antioxidant protein than did wild-type cells. Furthermore, we checked the mitochondrial protein synthesis rate in C150T mutant and wild-type cells by labeling the cell lines for 45 min with [^{35}S]methionine in methionine-free culture medium in the presence of emetine. The results showed that C150T mutant cells have a higher mitochondrial protein synthesis rate than wild-type cells. These results prompted us to check the protein level of COX by Western blots. The results showed that C150T mutant cells have higher levels of COX subunits I and II, which are encoded by mtDNA, whereas COX subunit IV, which is encoded by nuclear DNA, does not differ significantly in level between the

mutant and the wild-type cell lines. By contrast, the mRNA level of COX subunit I did not exhibit important differences between the two types of cell lines.

It is known that the frequency of certain mtDNA mutations may be dependent on the mtDNA haplogroup. Therefore, we checked the haplogroup of the C150T mutation-carrying mtDNA and of the mtDNA of the wild-type cells used for the aforementioned experiments. We found that the C150T mutant cells that we analyzed exhibited the U haplogroup and the non-mutant cells exhibited the J haplogroup. As the haplogroups were different, we searched through our stocks of fibroblast strains for a non-mutant strain of the U haplogroup and a C150T mutation-carrying strain of the J haplogroup. We have found these, and have made ρ^0 transformants from them. The biochemical analyses of these new cybrids are underway.

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191. Identification of a novel mitochondrial complex containing mitofusin 2 and stomatin-like protein 2

Petr Hájek, Anne Chomyn

A reverse genetics approach was utilized to discover new proteins that interact with the mitochondrial fusion mediator mitofusin 2 (Mfn2), and may participate in mitochondrial fusion. In particular, *in vivo* formaldehyde (FA) crosslinking of whole HeLa cells and immunoprecipitation with purified Mfn2 antibodies of SDS cell lysates led to the detection of a ~42 kD protein. This protein was identified by liquid chromatography and tandem mass spectrometry (LC/MS/MS) as stomatin-like protein 2 (Stoml2), previously described as a peripheral plasma membrane protein of unknown function associated with the cytoskeleton of erythrocytes (1). Immunoblot analysis with anti-Stoml2 antibodies showed that Stoml2 could be specifically immunoprecipitated with Mfn2 antibody either from FA-crosslinked and SDS-lysed cells or from cells lysed with digitonin. Subsequent immunocytochemistry and cell fractionation experiments fully supported the conclusion that Stoml2 is indeed a mitochondrial protein. Furthermore, demonstration of mitochondrial membrane potential-dependent import of Stoml2 accompanied by proteolytic processing, together with the results of sublocalization experiments, suggested that Stoml2 is associated with the inner mitochondrial membrane and faces the intermembrane space. Notably, FA crosslinking revealed a "ladder" of high molecular weight protein species, indicating the presence of Stoml2-Mfn2 hetero-oligomers. Knockdown of Stoml2 by the siRNA approach showed a reduction of the mitochondrial membrane potential, without, however, any obvious changes in mitochondrial morphology.

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Summary: Our laboratory has two general aims. One is to understand how the powerful transcription factor, NF- B, is controlled. The other is to develop a method of reprogramming immune function to allow the immune system to deal with diseases that normally elude it.

The work on NF- B extends studies that date back 20 years ago when we discovered this transcription factor. In the ensuing time, NF- B has emerged as a multi-potent factor able to activate many genes in many physiological settings but the primary function is in orchestrating inflammatory responses. Today, our main concern is with how such a plieotropic protein could exhibit the exquisite specificity of response that is characteristic of NF- B and how the body resolves NF- B activation episodes. The latter point is critical because NF- B activation leads to such a broad change in cellular behavior, even the generation of cancer, which it has to be under tight feedback control.

The work on feedback control involves the study of many proteins but most recently has led us to examine the possible role of microRNAs. In fact, we have found a number of microRNAs that are induced by inflammatory mediators and depend on NF- B for their synthesis. We are continuing this line of investigation, studying the mode of activation of one microRNA, miR-146, and the induction of others.

Our attempts to understand specificity have led us to investigate the pathways of NF- B activation. Most recently, we have taken that to the single cell level. This is technically very demanding but we now have the system under control and are starting to get data.

Another approach to specificity is to examine the various dimers that constitute the NF- B system. We have developed mutant NF- B subunits that form only certain complexes and are investigating their properties.

NF- B plays a critical role in non-inflammatory processes in the brain. There, NF- B is activated by glutamate. We are actively studying the biochemistry of this process.

Our work on reprogramming immune functions we call Engineering Immunity. This is a wide-ranging program involving studies of T cell function in cancer and B cell function in HIV infection. Our goals here are clinical but the problems we are investigating are molecular and cellular. We are collaborating with others on the clinical aspects. We have made significant progress in this area but it is still an early-stage program.

192. Dynamics of LPS and taxol-stimulated activation of NF- B

Markus Covert, David Baltimore

Last year, we found that the activation dynamics of the transcription factor NF- B exhibit damped oscillatory behavior when cells are stimulated by tumor necrosis factor α (TNFα) but stable behavior when stimulated by lipopolysaccharide (LPS). LPS binding to Toll-Like Receptor-4 (TLR-4) causes activation of NF- B that requires two downstream pathways, each of which when isolated exhibits damped oscillatory behavior. Computational modeling of the two TLR-4-dependent signaling pathways suggests that one pathway requires a time-delay to establish early anti-phase activation of NF- B by the two pathways. The MyD88-independent pathway required IRF-3-dependent expression of TNFα to activate NF- B, and the time required for TNFα synthesis established the delay. This work was published by *Science* last September.

There are some interesting questions raised from the work described above, which require single-cell analysis to answer. For example, we indicated that autocrine signaling plays a significant role in TLR-4-dependent NF- B activation. However, we have since found it difficult to detect a significant concentration of the autocrine signal. This raises the question, is the signal propagating outside of the cell? To answer this and other questions, we are looking at NF- B-related autocrine signaling at the single-cell level. We have cloned a p65-dsRed construct under the control of the endogenous p65 promoter into a lentiviral vector developed in the Baltimore lab, and have used time-lapse confocal microscopy to observe NF- B shuttling in and out of the nucleus under TNFα stimulation conditions. We will use this system to study NF- B localization under a variety of stimulation conditions and in a variety of knockout cells. We can use this system to observe propagation of the autocrine signal between cells. We will see if the signal radiates out evenly from the initiator cell, if it is polarized, or only strong enough to re-stimulate the same cell that secreted it. We can also grow the cells more sparsely to determine how close the neighboring cells need to be to receive the signal. From a computational side, we can obtain parameters that describe the propagation of the signal, in terms of diffusion, and indirectly of IRF-3 activation and TNFα synthesis.

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193. Dissecting the glutamate activation pathway for NF-κB

Chee Kwee Ea, David Baltimore

Nuclear factor κB (NF-κB) transcription factor is activated within the CNS in several forms of neuronal disease. Recent studies using a number of model systems clearly indicate an additional role of NF-κB in the normal physiological settings. NF-κB is present at synapses and is activated by glutamate. Genetic study has revealed a role for NF-κB in spatial learning. However, the molecular mechanism of glutamate-induced NF-κB activation is still unclear. This project will characterize the signaling pathway leading to NF-κB activation in response to glutamate by combining cell biology, biochemistry and microarray gene profiling. Specific aims are: 1) To determine the role of IKK in NF-κB activation by glutamate; 2) To identify the factor (GRAI) required for NF-κB activation by glutamate; and, 3) To determine genes that are regulated by NF-κB in response to glutamate. Understanding how glutamate leads to activation of NF-κB will provide fundamental insights into the regulation of NF-κB in the CNS under physiological and pathological conditions. Moreover, identification of NF-κB regulated genes in response to glutamate will shed light on spatial learning and memory.

194. Analysis of regulation and function of microRNA miR-146

Konstantin D. Taganov, Mark P. Boldin, and David Baltimore

Inflammation is a complex, highly regulated defense reaction orchestrated by the host in response to invading pathogen or injury. Innate immunity is the first line of defense, designed to recognize pathogen-associated molecular patterns represented by conserved components of microorganisms unique to microbial world. The pathogen molecules are sensed via a limited number of pattern-recognition receptors of the Toll receptor (TLR) family expressed in innate immune cells such as macrophages and dendritic cells. Stimulation of the TLRs by their respective ligands initiates appropriate responses leading to a production of variety of cytokines, which in turn shape and enhance the inflammatory and adaptive immune responses.

Activation of mammalian innate and acquired immune responses must be tightly regulated by elaborate mechanisms to control their onset and termination. MicroRNAs have been implicated as negative regulators controlling diverse biological processes at the level of posttranscriptional repression. Expression profiling of 200 microRNAs in human monocytes revealed that several of them (miR-146a b, miR-132, and miR-155) are endotoxin-responsive genes. Analysis of miR-146a and miR-146b gene expression unveiled a pattern of induction in response to a variety of microbial components and proinflammatory cytokines. By means of promoter analysis, miR-146a was found to be a NF-κB-dependent gene. Importantly, miR-146a/b were predicted to base-pair with sequences in the 3' UTRs of the TNF receptor-associated factor 6 (TRAF6) and IL-1 receptor-associated kinase 1 (IRAK1) genes, and we found that these UTRs inhibit expression of a linked reporter gene in a miR-146-dependent fashion. These genes encode two key adapter molecules downstream of Toll-like and cytokine receptors. Thus, we propose a role for miR-146 in control of Toll-like receptor and cytokine signaling through a negative feedback regulation loop involving down-regulation of IRAK1and TRAF6 protein levels.

195. Regulation of inflammation by the NF-κB system

Shengli Hao, David Baltimore

The inflammatory response is a double-edged sword. Whereas it is important for the host to clear out pathogenic insults and to repair injuries, an unregulated or prolonged inflammatory response leads to tissue damage which underlies a great number of diseases including autoimmune diseases such as type I diabetes, multiple sclerosis (MS), cancer, and vascular diseases. Therefore, the effective prevention and treatment of these diseases will be aided by a deep understanding of how the inflammatory response is controlled in the body. NF-κB is a key regulator of the inflammatory response. It functions as a homodimer or heterodimer drawn from a pool of five members: RelA, RelB, c-Rel, p50 and p52. It is the dimer, but not each NF-κB member, that determines the specificity of gene regulation. In addition, the NF-κB dimers have different functions in regulation of various aspects of the inflammatory response, and thus may contribute differently to the pathogenesis of the inflammation-associated diseases. Therefore, exploration of the specific properties and function of each dimer is crucial to understanding inflammation and ultimately to discover effective treatments for diseases of uncontrolled inflammation. However, this approach faces a huge obstacle because altering one NF-κB protein member affects all the dimers containing this member. To overcome this problem, we proposed in last year's annual report reconstructing NF-κB protein (using p50 as an example) such that this new NF-κB molecule (designated as p50*) can only form a homodimer with itself (p50*/p50*) but not with any wild type of NF-κB members. In order to approach this goal, we have used the following strategies to overcome various difficulties: 1) Since p50 lacks a transactivation domain, it is very

difficult to quantify the relative activity of a mutant, so we have solved this problem by fusing the transactivation domain of p65 to the C-terminus of p50, whose activity is greatly amplified and easily measured using a κB-luc reporter assay, and the relative activity of each p50 mutant can be readily detected and compared under the constant conditions; 2) With this easy, quick, sensitive, and quantitative tool, we have generated over 80 mutants of p50, concentrating on 10 amino acids at the dimerization interface, by site-directed mutagenesis. The relative importance of these residues in forming the dimer were therefore analyzed. With this knowledge, a mutant that perfectly matches our requirements has been created, which maintains the full activity of wild-type but does not associated with any other wild type NF-κB monomer proteins as detected by luciferase assay and co-immunoprecipitation. The same strategy has also applied to create a specific p50 and p65 mutant, each of which does not have any (or barely detectable) activity, thus does not function as a homodimer, but functions in the presence of the other as the p50:p65 heterodimer. Now we have a few p50 and p65 mutants that either only form p50 homodimer or p50:p65 heterodimer. The following approaches are under way currently: 1) Analyzing the role of p50 homodimer and p50:p65 heterodimer under various physiological contexts in different cell lines and primary animal cells; 2) creating p65 and p52 homodimer and other dimers using a similar strategy.

196. Engineering immunity to treat cancer

Lili Yang, Abigail Elliott, David Baltimore

The concept of Engineering Immunity is to reprogram the immune system so that it is able to handle diseases that the unperturbed system cannot deal with. To this end, we developed the ability to genetically program hematopoietic stem cells (HSCs), the common immune cell progenitors, to develop into T cells with the desired specificity *in vivo*. In a mouse tumor model, we were able to show that this method could generate large number of anti-tumor T cells that resulted in efficient tumor resistance and eradication (Ref. #1). To further advance and mature the method, we have been actively working on the following areas:

a. Engineering immunity to treat melanoma

In July 2005, we started collaboration with several research and clinical groups at UCLA, USC, CHLA and UCHC to move the Engineering Immunity method into clinical trials to treat melanoma. A Phase I clinical trial is planned for launch in 2007 at UCLA, conducted by Dr. James Economou's clinical team.

b. Generation of antigen-specific T cells with enhanced function *in vivo*

As an extension of our Engineering Immunity concept, we have been working on the generation of antigen-specific T cells with enhanced function *in vivo*. Taking advantage of our lab's extensive experience with engineering viral vectors, we are able to construct vectors that efficiently co-express multiple genes under the control of a single promoter. This technique allows us to put into one vector the two genes encoding an antigen-specific TCR, together with a regulatory gene that can endow T cells with a desired function. One such gene we tested is that encoding IL-15, which has been considered to play an important role on CD8 cytotoxic T cell homeostasis and memory. We are in the process of testing the anti-tumor effect of these genetically enhanced T cells.

c. Targeted gene delivery to genetically modify HSCs *in vivo*

As a hallmark of gene therapy, targeted gene delivery *in vivo* can greatly facilitate the application of gene therapy, including our "Engineering Immunity" strategy. To this end, we have developed a gene delivery system to transfer genes to chosen cell types (Ref. #2). In particular, we have generated lentiviruses that can specifically infect cells expressing mouse c-kit, a marker for mouse HSCs; and human CD34, the sole reliable marker for human HSCs. We have proved this system to be highly specific and efficient, by targeted infection of cell lines expressing the c-kit or CD34 marker. The targeted infection of primary mouse HSCs and human HSCs are under testing, both *in vitro* and *in vivo*. Other applications of this system include the *in vivo* correction of blood cell genetic deficiencies, holding potential for treating diseases such as SCID and the X-linked autoimmunity.

d. *In situ* dendritic cell vaccination

Dendritic cell (DC) vaccination has been broadly used to mount T cell immunity against cancer and infectious diseases. Genetically modified DCs that express tumor or viral antigens are considered to be powerful vaccines. However, the current protocol involves a costly and tedious process including collecting peripheral blood from a patient, culturing DCs *in vitro*, tranducing the cells with vectors, and infuse the cells back to the same patient. Falling into the category of personal medicine, the process is costly, tedious and likely inconsistent. We developed a novel method of *in situ* DC vaccination. Our strategy is to engineer the tropism of a lentiviral vector to make it infect only DCs, but not any other tissue cells. Using Chicken Ovalbumin (OVA) as a model antigen, we were able to induce a strong anti-OVA T cell response and memory in a mouse via a single shot of such lentivector encoding OVA. What's more, by modifying the expression pattern of OVA, we can also induce a strong antibody response in mouse. In the tumor challenge experiments, all mice receiving such a vaccination showed complete resistance to OVA tumor challenge. Currently, we are in testing of the method for authentic tumor antigen, in a B16 mouse melanoma model. This new method could potentially be used to replace the current DC vaccination approaches, by a single shot of a common lentivector stock, to treat a broad range of diseases including cancer and HIV.

197. Engineering immunity to treat HIV and other dangerous pathogens

Xin Luo, Ryan Michael O'Connell, Angie Frausto, Dongzi Yu, Lili Yang, David Baltimore,* (*Lead Scientist and Project Manager)

Supported by Bill and Melinda Gates Foundation through the Grand Challenges in Global Health Initiative, we are exploring a fundamentally new way of stimulating the immune system to fight off infectious diseases, focusing on HIV as a test of the concept. The promise of this project is that for some infections, including HIV, the immune system's natural responses are inherently inadequate, and therefore, the traditional approach of using vaccines to stimulate and boost these responses is likely to be ineffective. As an alternative, we propose to genetically engineer immune cells so that they can produce an adequate responses. Our work is intended to eventually lead to immunotherapy for people who are infected with HIV. It could also lead to new ways to prevent HIV infection.

Our strategy is to use gene therapy for expression of genes encoding neutralizing antibodies or antibody-like proteins against HIV. Multiple problems must be solved to allow expression of purposely-designed genes in the cells of infected people. We plan to implant genes in blood stem cells and allow the cells to give rise to B cells, the body's natural antibody-producing cells, requiring the solution of particular problems posed by the architecture of antibody genes. We must also design antibodies or antibody-like proteins that can efficiently neutralize the infectivity of HIV. This will be done by design methods or by selection. We must then prove the effectiveness of the design in human cells or in a mouse/human chimera. Finally, we have to drive the cost of the process to a low enough level to use in the less developed world. At low cost, it could even be a vaccine.

With the goal of developing this general method, captured by the phrase "Engineering Immunity" to treat infection by HIV and other dangerous pathogens, we have the following objectives:
(Note: This project is a joint effort led by the Baltimore group, and joined by Dr. Pamela Bjorkman's group at Caltech, and Dr. Pin Wang's group at USC.)

Objective 1: To perfect lentiviral expression systems that can impart the desired specificities to the murine Ab repertoire. (Lili Yang, Dongzi Yu)

The Baltimore laboratory has devised methods to generate functional T cells of defined antigen specificity in mice by retrovirus-mediated expression of T cell receptor genes in hematopoietic precursor cells (Ref. #1). We will adapt these methods to generate functional B cells capable of secreting antibodies (Abs) or Ab-like proteins upon stimulation with antigen. Using a model antibody/antigen system, we will construct lentiviral vectors that co-express a membrane-bound form of a lysozyme-specific Ab, and a secreting form of anti-HIV neutralizing Abs (Nab). Upon transferring into HSCs, such a lentivector will direct the generation of B cells in mouse that can go through clonal expansion upon injection of lysozyme, and will secrete high titers of anti-HIV Abs. So far we have achieved the generation of lysozyme-specific B cells in mouse. Functional analysis of these B cells in response to antigen stimulation *in vitro* and *in vivo* is ongoing.

Objective 2: To engineer bispecific and other designer anti-HIV antibodies and evaluate them for binding to and neutralizing HIV. (This objective is performed by Dr. Pamela Bjorkman's group, Division of Biology, Caltech)

Using protein engineering and design methods, we will increase Ab affinities to allow them to be effective at low concentrations in the blood or in secretions, and make bispecific Abs containing two different combining sites. We will express, purify and determine affinities of the following Abs and Ab-like proteins: bispecific antibodies with specificities for two different HIV epitopes, bispecific antibodies with one HIV specificity and one host immune effector cell specificity, chimeric proteins containing the gp120-binding domains of CD4 linked to a CD4-induced antibody specificity, multivalent antibodies (triabodies or tetravalent antibodies). Also, we will increase affinities of Abs or Ab-like proteins using phage display or computational protein design methods. Function of the designer anti-HIV Abs and Ab-like proteins will be evaluated using an *in vitro* neutralization assay.

After obtaining DNA encoding the broadly neutralizing anti-HIV antibodies 4E10, 2F5, 2G12, and b12, we have proceeded to construct expression plasmids for this starting set of NAbs in several forms: full-length IgG, Fab, and single-chain Fv (scFv). We are now in the process of expressing and purifying the IgG and Fabs using the baculovirus expression system. These reagents represent the foundation for future work on the project in two ways: (1) they provide benchmarks for comparison of future engineered reagents; and (2) the scFv and Fab forms are the building blocks that will be combined or mutated to form improved reagents.

Objective 3: To test anti-HIV-producing lentiviral constructs *in vitro* using a B lymphopoiesis culture system and *in vivo* using human/mouse chimeras. (Xin Luo, Ryan Michael O'Connell, Angie Frausto)

The overall goal of this objective is to generate and evaluate at least five optimized lentiviral vectors, each of which will encode a different human anti-HIV NAb or Ab-like protein. We will perfect an *in vitro* B lymphopoiesis culture system and creating human/mouse chimeras that have been reconstituted with a human immune system. These will be used to test lentiviral constructs. We will also construct anti-HIV producing lentiviral systems, with an initial focus on engineering "dual specificity" B cells that will secrete desired anti-HIV Abs or Ab-like proteins upon vaccination with a linear peptide antigen. Our goal is to produce lentiviral constructs that will drive transduced $CD34^+$ human cord blood cells to differentiate into human B cells with dual

specificity in the *in vitro* lymphopoiesis culture system and *in vivo* in the human/mouse chimeras.

Objective 3a: To establish an in vitro B lymphopoiesis culture system **(**Xin Luo, Angie Frausto)

Based on the reported long-term, *in vitro* culture system initiated with $CD34^+$ human cord blood hematopoietic progenitor cells that supports normal human B-lineage development (Fluckiger *et al*., 1998), we have started a two-stage system in order to perfect the conditions required for B lymphopoiesis *in vitro* and subsequently, for future testing of lentiviral constructs that drive the transduced human HSCs to differentiate into human B cells with dual specificity *in vitro*. The first stage is to generate an adequate supply of $CD19^+$ B cells from $CD34^+$ human hematopoietic progenitor cells. Using a MS-5 stromal cell line for the co-culture system in the presence of growth factors, we have achieved the generation of CD19+ cells. We further proved that we can transduce the CD34+ cells with a reporter lentivector at high efficiency (>80%), and differentiate the transduced cells into CD19+ cells. The second stage is to activate naïve $CD19^+$ B cells and differentiate them into Ab-producing plasma cells. By stimulating naïve B cells with mitogens in the presence of T cell help, we observed the class switching and upregulation of B cell activation markers. Future work will be to further perfect the culture system and use it to test the candidate lentiviral vectors for directing B cell development and mediating production of specific proteins.

Objective 3b: To establish a human/mouse chimeras model **(**Ryan Michael O'Connell)

Manz and co-workers have reported that intrahepatic injection of human $CD34^+$ cord blood cells in newborn $RAG2^{-/-}\gamma_c^{-/-}$ mice results in reconstitution of a human adaptive immune system in mice (Traggiai *et al*., 2004). The transplanted mice develop human B, T and dendritic cells, generate structured primary and secondary lymphoid organs, and establish a functional immune response. This could provide us with a convenient *in vivo* model with which we can evaluate the efficacy of the genetically programmed human HSCs to develop into functional B cells. We have obtained the $RAG2^{-/-}\gamma_c^{-/-}$ mouse strain, and have located a source for human cord blood cells. We are now starting to establish this human/mouse chimeras model for testing candidate lentivectors.

Objective 4: To develop a novel murine model of HIV infection and to test the efficacy of the anti-HIV engineered B cells to prevent and clear HIV infection *in vivo*.

To test our Engineering Immunity approach, we will develop a new mouse model of HIV pathogenesis involving human/mouse chimeras that have been reconstituted with a human adaptive immune system and infected with HIV.

Objective 5: To engineer the lentivector delivery system for the application of Engineering Immunity approach. (This objective is performed by Dr. Pin Wang's group at USC's Mork Family Department of Chemical Engineering and Materials Science.)

We have identified an efficient and flexible method to target lentivirus-mediated gene transduction to a pre-determined cell type (Ref. #2). It involves incorporation of a cell-specific binding determinant (CBD) and a fusogenic protein as two distinct entities into the lentiviral surface. The fusogen is modified so that it will not bind to its cognate receptor, so the CBD determines the targeting specificity. Using CD20 as a target antigen for human B cells, we have demonstrated that this targeting strategy is effective both *in vitro* and in intact animals. This methodology is flexible and can be extended to other forms of cell-type specific recognition to mediate targeting. The only requirement is that the antibody (or other binding protein) must be endocytosed after interaction with its cell-surface binding determinant. The technique allows for targeting of gene therapy by direct injection of a vector.

CD34 is the sole reliable marker for human HSCs. We plan to use our new method to target lentiviral vectors to CD34-positive cells. We have cloned out an anti-CD34 antibody that can induce endocytosis upon binding to CD34, and have shown that lentivector enveloped with this antibody can efficiently infect a stable 293T cell line that expresses CD34. Transduction efficacy of the lentivector to primary human CD34 cells *in vitro* in cell culture, and *in vivo* in the RAG2-/-$\gamma_c^{-/-}$ human mouse chimeras is under testing.

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Summary: My laboratory is interested in protein-protein interactions, particularly those mediating immune recognition. We use X-ray crystallography and biochemistry to study purified proteins **(Figure 1),** and are beginning to include confocal and electron microscopy to examine protein complexes in cells. Some of our work focuses upon homologs and mimics of class I MHC proteins. These proteins have similar three-dimensional structures, but different functions including immune functions (IgG transport by the neonatal Fc receptor, FcRn, and evasion of the immune response by viral MHC mimics), and non-immune functions (regulation of iron or lipid metabolism by HFE and ZAG, and serving a chaperone function for pheromone receptors in the case of the M10 proteins). We are also comparing the structures and functions of host and viral Fc receptors with FcRn.

Transfer of maternal IgG molecules to the fetus or infant is a mechanism by which mammalian neonates acquire humoral immunity to antigens encountered by the mother. The protein responsible for the transfer of IgG is the MHC class I-related receptor FcRn. MHC class I molecules have no reported function as immunoglobulin receptors; instead they bind and present short peptides to T cells as part of immune surveillance to detect intracellular pathogens. We solved the crystal structures of rat FcRn both alone and complexed with Fc. We are now interested in using information obtained from our crystallographic and biochemical studies to address how FcRn-IgG complexes are transported across polarized epithelial cells. We are using a combination of confocal and electron microscopy to study the itineraries of FcRn-containing endosomes in transfected epithelial cells and in the proximal small intestine of neonatal rats. We are also doing structure/function studies of other Fc receptors that are not MHC homologs: gE/gI, a viral Fc receptor for IgG, FcαRI, a host receptor for IgA, and the polymeric immunoglobulin receptor (pIgR), a receptor that transports dimeric IgA and polymeric IgM into secretions.

HFE is a recently discovered class I MHC homolog that is involved in the regulation of iron metabolism, an unexpected function for an MHC-related protein. The gene encoding HFE is mutated in patients with the iron overload disease hereditary hemochromatosis. HFE has been linked to iron metabolism with the demonstration that it binds to transferrin receptor, the receptor by which cells acquire iron-loaded transferrin. We solved crystal structures of HFE alone and HFE bound to transferrin receptor. The interaction of HFE with transferrin receptor is a fascinating system to study because we can use crystal structures to answer biochemical, functional, and evolutionary questions that address how binding of HFE interferes with transferrin binding, if conformational changes in the receptor are involved in the binding of either transferrin or HFE, which part of the MHC-like HFE structure binds transferrin receptor, and how the HFE interaction with the receptor compares with interactions of ligands with MHC and MHC-like (e.g., FcRn) proteins. We are expanding our studies to include cell biological investigations of HFE and transferrin receptor intracellular trafficking in transfected cell lines using confocal microscopy and other imaging techniques.

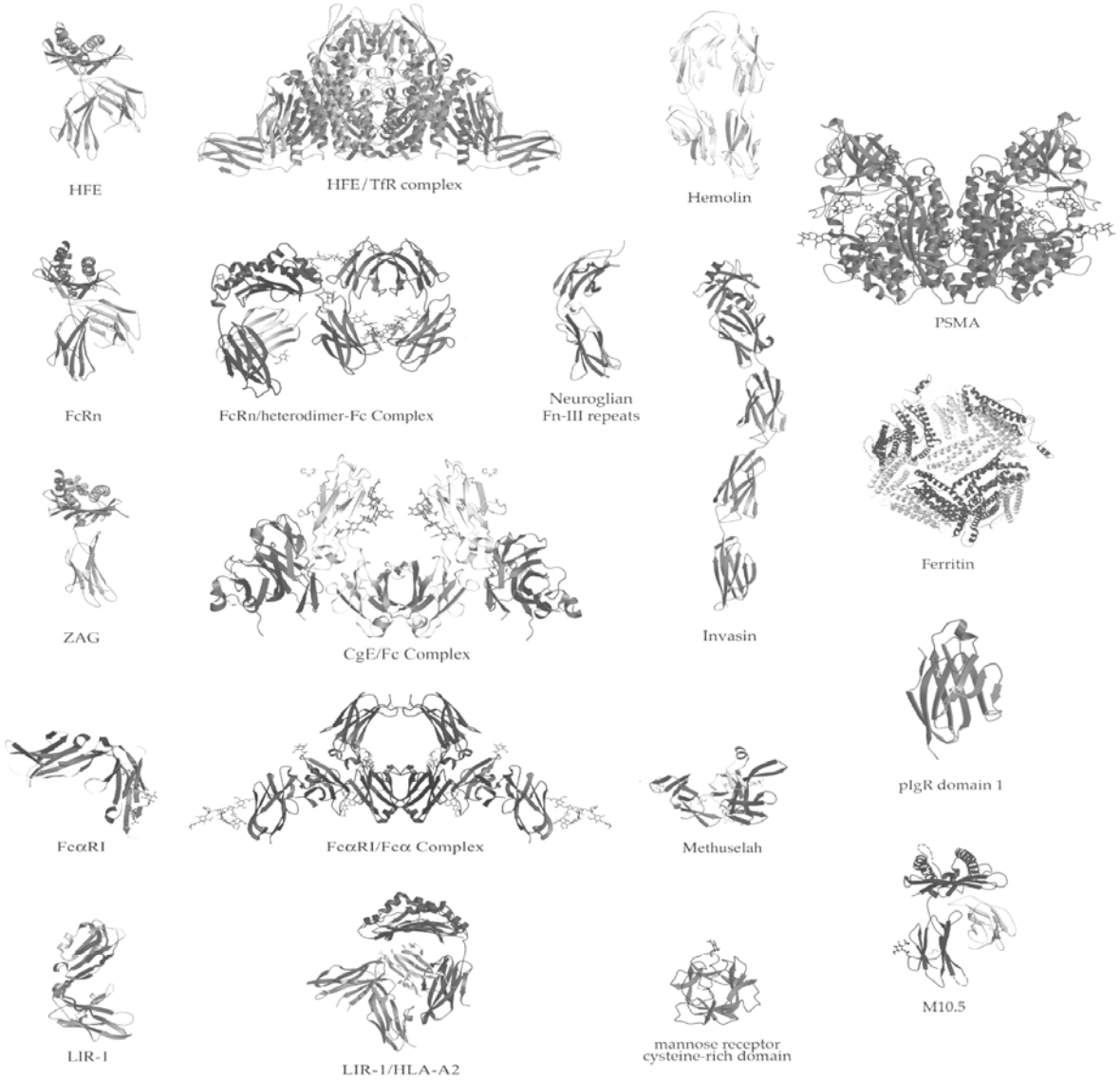
We are also interested in other MHC homologs, including proteins encoded by viruses. Both human and murine cytomegalovirus (HCMV, MCMV), express relatives of MHC class I heavy chains, probably as part of the viral defense mechanism against the mammalian immune system. Our biochemical studies show that the HCMV homolog associates with endogenous peptides resembling those that bind to class I MHC molecules. Our current efforts focuses upon defining the structure and function of these homologs in order to understand why viruses make them and how they interfere with the host immune system.

Recent studies from other laboratories have revealed expression of an interesting family of class Ib MHC proteins (M10s) that interact with putative pheromone receptors in the rodent vomeronasal organ. This interaction may play a direct role in the detection of pheromonal cues that initiate reproductive and territorial behaviors. We have solved the crystal structure of M10.5, an M10 family member. The structure shows that M10.5 folds into a similar structure as a *bona fide* class I MHC molecule. Unexpectedly, however, the M10.5 counterpart of the MHC peptide-binding groove is open and unoccupied, revealing the first structure of an empty class I

MHC molecule. Our biochemical data suggest that M10.5 associates with some sort of groove occupant, most likely non-peptidic. The challenge now is to discover the physiological ligand(s) of M10 proteins and understand how they associate with pheromone receptors to influence mating behaviors.

Our structural work on class I MHC homologs has elucidated new and unexpected recognition properties of the MHC fold. For FcRn and HFE, the structural and biochemical studies have revealed a similar fold and some common properties, including the assumption that both receptors "lie down" parallel to the membrane when binding ligand, and a sharp pH-dependent affinity transition near neutral pH. In the case of FcRn, we have elucidated the structural basis of its pH-dependent interaction with IgG and will now focus upon cell biological studies of intracellular trafficking, for which the pH-dependent interaction is critical.

The pH dependence of the HFE-TfR interaction suggested to us that intracellular trafficking studies of HFE would be interesting, so much of our future efforts on both the FcRn and HFE systems will center around probing their function in a cellular context using imaging techniques. Our functional studies of M10 proteins are just beginning, since we do not understand the nature of the interaction between an M10 protein and a pheromone receptor and how that relates to binding to an as yet unidentified groove occupant. We are at an even earlier stage in our studies of the viral MHC homologs, in which our primary goal will be to solve crystal structures of a viral homolog alone and complexed with its cellular receptor.



198. Effects of ligand valency on FcRn-mediated transcytosis

Devin B. Tesar, Noreen Tiangco

The neonatal Fc receptor (FcRn) transports IgG across epithelial cell barriers to provide maternal antibodies to offspring and serves as a protection receptor by rescuing endocytosed IgG and albumin from lysosomal degradation. We have generated polarized Madin-Darby Canine Kidney (MDCK) cell lines expressing rat FcRn (rFcRn) to investigate the potential requirement for ligand bivalency in FcRn-mediated transport. The rFcRn-MDCK cells bind, internalize, and bidirectionally transcytose the bivalent ligands IgG and Fc across polarized cell monolayers. However, they cannot be used to study FcRn-mediated transport of the monovalent ligand albumin, as we observe no specific binding, internalization, or transcytosis of rat albumin at concentrations up to 10 μM. To address whether ligand bivalency is required for transport, the ability of rFcRn to transcytose or recycle wild-type Fc homodimers (wtFc; two FcRn binding sites) and a heterodimeric Fc (hdFc; one FcRn binding site) was compared. We have shown that ligand bivalency is not absolutely required for transcytosis or recycling to occur, but that wtFc is transcytosed and recycled more efficiently than hdFc, particularly at lower concentrations. We have further shown hdFc and wtFc have different intracellular fates, with more hdFc than wtFc being trafficked to lysosomes and degraded, suggesting a role for avidity effects in FcRn-mediated IgG transport.

199. Characterization of FcRn-mediated transport pathways via confocal microscopy

Devin B. Tesar, Galina Jerdeva

Movement of specific protein molecules across epithelial cell barriers by their cognate receptors is achieved via a multivesicular transport pathway known as transcytosis. Discrete steps in the procession of a receptor-bound ligand through the transcytotic network are characterized by association with distinct subpopulations of endosomal compartments. These subpopulations of endosomes can be identified by confocal microscopy using fluorescent markers (such as transferrin) or antibodies against such markers (such as anti-Rab antibodies). We are currently working to decipher the transcytotic itinerary of FcRn, with or without its IgG ligand, by colocalizing FcRn and IgG with different endosomal markers. To achieve this, FcRn can be visualized by expressing a C-terminal GFP-fused form of FcRn, or by staining with an anti-FcRn monoclonal antibody that is directly conjugated to Alexa488. Rat IgG or Fc fragment can be directly labeled with fluorescent dyes (such as RITC or Cy3), and antibodies against specific endosomal markers can be viewed using secondary antibodies conjugated to a far-red dye (such as Cy5). This allows for three-color confocal analysis to determine the intracellular localization of FcRn, it ligand, and marker proteins for particular compartments at different stages within the transport process. These data can then be compared to the transport pathways of more well characterized receptors such as the polymeric Ig receptor (pIgR) to evaluate which steps might be unique to, or particularly important during FcRn-mediated transport of IgG. Future work will focus on developing techniques for following trafficking events in live cells over the course of several minutes in real time.

200. Identification of effector molecules that mediate the trafficking of FcRn in cells

Devin B Tesar, Johannes Graumann[*]

The intracellular trafficking of a receptor and its ligands requires the coordinated association of the receptor cytoplasmic tail with effector molecules responsible for regulating and executing specific steps of the trafficking pathway. Determinants within the cytoplasmic tail of a given receptor confer the specificity of these interactions. While many of the molecular components of the cytosolic trafficking machinery are very well conserved across different organisms, different receptors may utilize different components while being directed to their target destinations. We wish to determine the identity of effector proteins associated with FcRn during its intracellular trafficking itinerary. FcRn obtained from both natural sources (neonatal rat intestine) and tissue culture cells (transfected MDCK cells) is co-immunoprecipitated using FcRn-specific antibodies, and the co-precipitating proteins are subjected to Multidimensional Protein Identification Technology (MudPIT) analysis. MudPIT allows mass spectrometric analysis of a complex protein mixture by coupling a two-dimensional chromatographic separation of the mixture to direct downstream injection of the sample into the mass spectrometer. Identified targets will be verified by performing pull downs of the identified binding partner and demonstrating the co-precipitation of FcRn. Further experiments will then be carried out to assess the role of these targets in FcRn-mediated intracellular trafficking.

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201. Characterization and visualization of the FcRn-dependent transcytotic pathway using high-resolution fluorescence confocal microscopy

Galina V. Jerdeva, Devin B. Tesar, Scott E. Fraser[*]

Specific delivery of proteins across polarized epithelia is controlled by receptor-mediated transcytosis. Based on studies of the trafficking of model receptors such as the polymeric immunoglobulin receptor (pIgR), the pathways for receptor-mediated transport of protein ligands in the basolateral to apical direction are relatively well understood. Less is known about the trafficking pathways for receptors that transport from the apical to basolateral surface. One such receptor is the neonatal Fc receptor (FcRn), which transports maternal immunoglobulin G (IgG) across intestinal or placental epithelial barriers to provide immunity to fetal or newborn mammals and serves as a protection receptor for IgG, thereby increases its serum half-life. To investigate FcRn-mediated transport of IgG, we are using Madin-Darby

Canine Kidney (MDCK) cells stably expressing FcRn (MDCK-FcRn). The transfected cells specifically transcytose IgG and Fc across polarized cell monolayers, therefore presenting an *ex vivo* system that mimics the *in vivo* FcRn-dependent transport system. Using high-resolution confocal and multiphoton microscopy, we will identify intracellular compartments involved in transcytosis of labeled Fc and IgG in MDCK-FcRn cells by colocalization studies with organelle-specific markers. Comparing the intracellular trafficking of FcRn and its ligands with trafficking of a basal to apical model receptor, pIgR, which transports dimeric dIgA (dIgA), will provide information about common and specific trafficking pathways of IgG versus dIgA. To compare FcRn- and pIgR-dependent intracellular pathways**,** we will generate MDCK cells stably expressing both receptors (MDCK-FcRn-pIgR). Fluorescently-labeled Fc and dIgA will be internalized apically and basolaterally, respectively, by polarized MDCK-FcRn-pIgR monolayers, and the intracellular locations of internalized ligands will be investigated by confocal or multi-photon microscopy. To get more detailed information about the dynamics of Fc trafficking and the identities of trafficking compartments, real-time high-resolution microscopy will be performed on living polarized cells using highly efficient, photo-bleaching resistant fluorescent probes such as Alexa-dyes and Quantum Dots.

[*]*Professor, Division of Biology, Caltech*

202. Three-dimensional pathways of transport maternal antibody across the epithelial barriers in suckling rat small intestine revealed by electron tomography

Wanzhong He, Christine Kivork, Mary Morphew[*], J. Richard McIntosh[*], Grant Jensen[**]

Before its own immune system is fully developed, the suckling rat is dependent upon the neonatal Fc receptor (FcRn) to transport maternal IgG from ingested milk into its bloodstream. FcRn also functions to protect IgG from lysosomal degradation. In both its transport and protection roles, FcRn binds to IgG at acidic pH (6.0) inside endosomes and releases IgG at the pH (7.4) of blood. Early conventional electron microscopy studies using labeled IgG provided direct evidence that the proximal small intestine of neonatal rat selectively transports antibody into the circulation. However, due to the large size and non-specific coupling of the labels that were used (e.g., ferritin), not all of the labeled IgG is likely to bind FcRn, thus these studies may not reveal the true pathways of IgG uptake in intestinal epithelial cells. In order to trace the precise three-dimensional (3D) pathways during dynamic endocytic processes and to examine possible configurations of FcRn-Fc complexes within different types of intracellular vesicles in the intact neonatal rat small intestine, we need technique that permits us to trace fully functional antibody conjugates in an *in vivo* system with high resolution. We are 1.4 nm nanogold clusters, which are small chemically-defined particles, covalently attached to the hinge region of rat Fc then purified on an FcRn affinity column, as a more reliable label to explore functional endocytic processes. However, it is still challenging to visualize 1.4 nm gold particles within thin sections of tissue samples. Therefore, we are combining techniques: improved gold-enhancement methods for chemically-fixed samples, a newly developed freeze substitution (FS)-based silver-enhancement method for high pressure frozen samples, and electron tomography to visualize the gold particles and trace the precise pathways of antibodies in 3D. Our results clearly demonstrate that the proximal small intestine takes up the antibodies through an FcRn-specific mechanism with most gold particles located in tubular vesicles and coated vesicles, and discharges the conjugates to the basolateral extracellular space. Only a small portion of conjugates are found in lysosomes. In the distal small intestine, which does not express FcRn, we find the gold particles are delivered to the lysosomal degradation pathway. Hence, these techniques are confirmed to be very useful to trace 3D transport pathways and to analyze dynamic events in the process of transcytosis.

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203. Crystal structure of the HSV-1 Fc receptor bound to Fc reveals a mechanism for antibody bipolar bridging

Elizabeth R. Sprague, Chu Wang[*], David Baker[*]

Herpes simplex virus type-1 (HSV-1) expresses a heterodimeric Fc receptor, gE-gI, on the surfaces of virions and infected cells that binds the Fc region of host immunoglobulin G (IgG) and is implicated in the cell-to-cell spread of virus. gE-gI binds IgG at the basic pH of the cell surface and releases it at the acidic pH of lysosomes, consistent with a role in facilitating the degradation of anti-viral antibodies. Here we identify the C-terminal domain of the gE ectodomain domain of gE (CgE) as the minimal Fc-binding domain and present a 1.78 Å CgE structure. A 5Å gE-gI/Fc crystal structure, which was independently verified by a theoretical prediction method, reveals that CgE binds Fc at the C_H2-C_H3 interface, the binding site for several mammalian and bacterial Fc-binding proteins. The structure identifies interface histidines that may confer pH-dependent binding and regions of CgE implicated in cell-to-cell spread of virus. The ternary organization of the gE-gI/Fc complex is compatible with antibody bipolar bridging, which can interfere with the anti-viral immune response.

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204. Intracellular trafficking of an antibody bipolar bridged complex of HSV-1 Fc receptor-antiviral IgG

Alex Farley

Herpes Simplex Virus (HSV) is a model member of the alphaherpesvirus family, which also includes Varicella-Zoster Virus (ZVZ) and Pseudorabies Virus (PrV). Alphaherpesviruses are characterized by a relatively short replicative cycle in epithelial tissues and egression to and latent infection of the sensory neurons. HSV encodes two type 1 membrane-bound glycoproteins which together function as a receptor for the Fc portion of IgG. The heterodimer is composed of glycoprotein E (gE) and glycoprotein (gI) and it is found on the surface of virions and infected cells. The Fc receptor function of gE-gI is thought to provide a mechanism for immune evasion by blocking the effector function of host Fc recognition proteins in both the adaptive and innate immune systems. The gE-gI heterodimer is thought to bind to antigen-bound IgG in a process called bipolar bridging in which the antigen-binding fragments (Fabs) bind to an antigen, and gE-gI binds to the Fc. This process could provide a mechanism for HSV-1 to evade antibody-mediated immune responses via Fc receptors. The binding affinity of gE-gI for Fc is pH-dependent, whereby gE-gI binds IgG with high affinity at the slightly basic pH of the cell surface (~7.4) and releases IgG at acidic pH (≤6.4). The sharply pH-dependent binding suggests that IgG that is endocytosed by gE-gI dissociates from gE-gI at the low pH of endosomal compartments, where it could be degraded, while gE-gI is recycled back to the cell surface. Our hypothesis is that Fc binding to gE-gI results in endocytosis of the antibody/antigen complex and its consequent degradation possibly in the lysosome. We have shown that the gE-gI heterodimer can internalize the Fc domain of IgG. Future work will include experiments to address the intracellular trafficking patterns of gE-gI and the fates of gE-gI/IgG and gE-gI/IgG/antigen complexes.

205. Structural studies of the human cytomegalovirus immunoglobulin G receptors

Elizabeth R. Sprague, Alex Farley, Evelyn Cheung, Hartmut Henge[*]

We have expanded our work on viral Fc receptors to include the recently cloned Fc receptors from HCMV, which is a member of the betaherpesvirus family. HCMV encodes two glycoproteins, gp34 and gp68, that bind the Fc region of IgG; however, unlike gE-gI, the HCMV Fc receptors are composed of a single polypeptide whose sequence is unrelated to either gE or gI. We have expressed the ectodomain of gp68 in baculovirus-infected insect cells and purified milligrams of the protein. Using an analytical gel filtration assay to investigate binding of one of these receptors to Fc, we have shown that the recombinantly-expressed ectodomain of gp68 binds to the Fc portion of human IgG1 with a stoichiometry of two gp68 molecules per one Fc homodimer. Furthermore, we localized the gp68 binding site to the C_H2-C_H3 hinge region of Fc, which is where gE-gI binds, using Fc molecules where residues in this region were mutated in either one or both of the Fc subunits, analogous to our studies of the gE-gI/Fc interaction. In a surface plasmon resonance-based binding assay, the affinity of gp68 for Fc was measured as ~300 nM, and preliminary data suggest that unlike gE-gI/Fc, the gp68/Fc complex is unaffected by small changes in pH near neutral, indicating a possible divergent function between betaherpesvirus and alphaherpesvirus Fc receptors. We are undertaking crystallization of gp68 (wild-type and glycosylation variants) alone and bound to Fc. For gp34, we do not see expression of the gp34 ectodomain in baculovirus-infected insect cells, whereas we do see expression in CHO cells. The gp34 from CHO cell supernatants binds IgG; however, because it appears to be oligomerized, it is not ideal for further characterization at this time.

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206. Biochemical and structural studies of ferroportin

Adrian E. Rice, Douglas C. Rees[*]

All known organisms, save two bacteria, require iron for survival. Despite its importance, iron in overabundance is toxic. In order to maintain a balance of iron levels, organisms have developed a highly specialized network of molecules designed to monitor and maintain iron homeostasis. When the fidelity of these networks is compromised, diseases such as iron deficiency and iron overload result. Mammals lack any regulated mode of iron excretion and therefore must have highly regulated mechanisms for controlling the acquisition of iron from the diet. The primary site of iron absorption is the duodenum of the small intestine. This process can be categorized by two phases: 1) iron uptake across the brush border into the cytoplasm of duodenal enterocytes; and 2) iron export across the basolateral membrane of these cells into the blood. Iron from the diet is reduced from Fe3+ to Fe2+ by the membrane-bound iron reductase Dcytb and is transported across the apical brush border by an integral membrane protein called divalent metal transporter 1 (DMT1; also known as DCT1 and NRAMP2). Iron is then transported across the cell to the basolateral side where it is exported by the basolateral integral membrane iron transporter ferroportin (Fpn; also known as IREG1 and MTP1). Fpn is the only identified iron exporter in vertebrates and is an integral membrane protein containing 9-12 predicted alpha-helical transmembrane segments. Point mutations in Fpn lead to an autosomal dominant iron overload disease called ferroportin disease. Our project aims to characterize Fpn from both a structural and biochemical standpoint. Current plans in our laboratory aim at obtaining purified recombinant Fpn for these studies.

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207. Characterization of hemojuvelin and its interaction with neogenin

Fan Yang, Anthony P. West, Jr., Anya E. Wyman, An Sheng Zhang[*]*, Caroline A. Enns*[*]

Mutations in HJV, the gene encoding hemojuvelin, cause juvenile hemochromatosis, an iron overload disorder that affects young people of both sexes. Recent studies have showed that HJV might function through affecting the transcription of hepcidin, the key iron regulatory peptide. How this is achieved is not clear yet. Recent advances in Caroline Enns lab at OHSU found that hemojuvelin co-immunoprecipates with neogenin, a cell surface receptor belonging to the immunoglobulin (Ig) family. Based on previously published data indicating that the six fibronectin type III (FNIII) domains of neogenin binds to RGM, a close relative of hemojuvelin, we expressed both neogenin ectodomain and neogenin FNIII domains and found they have nearly identical affinities for hemojuvelin. Several proteolysis experiments were performed using neogenin FNIII domains as substrates and products were analyzed by pull-down assays followed by SDS/PAGE, Western blotting and N-terminal sequencing. We were able to narrow down the hemojuvelin-binding epitope of neogenin to two of the six FNIII repeats. Binding data obtained by a biosensor assay confirmed high affinity binding between neogenin and the isolated two FNIII domain fragment. Crystallization trials of hemojuvelin alone and complexed with the neogenin fragment have been initiated with the goal of obtaining high-resolution structural information about the interaction.

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207. Structural and functional studies of MHC class I homologs in HCMV

Zhiru (Jenny) Yang

HCMV affects 70-90% of all human populations and can be life threatening to immuno-compromised individuals, such as cancer, transplant, and AIDS patients. HCMV achieves its lifelong infection in host cells by adopting multiple mechanisms to evade the primed immune system, including down-regulation of host class I MHC molecules. HCMV encodes two MHC class I homologs, UL18 and UL142, which may be components of a strategy to avoid immune detection of class I-MHC negative cells. UL18 is heavily glycosylated (13 potential N-linked glycosylation sites) and able to bind host-derived β2-microglobulin (the class I MHC light chain) and endogenous peptides. The host cell receptor for UL18 is LIR-1, which is most abundant in B cells, monocytes, macrophages, and dendritic cells. The UL18/LIR-1 interaction is likely to contribute to the latency and persistence of HCMV as well as the viral evasion of host NK cell surveillance. In addition to UL18, LIR-1 also binds a broad range of host MHC class I molecules, but with an affinity that is over 1000 times reduced compared to its affinity for UL18[1]. The structures of LIR-1 and a LIR-1/HLA-A2 complex have recently been solved in our lab[2,3]. We are working on the co-crystallization and structure determination of UL18 with LIR-1. We have generated deglycosylated mutants of UL18, Crystals of mutant UL18/LIR1 complexes have been obtained, and we are now optimizing the crystallization conditions to get better diffraction. These studies will be extended to include a newly identified HCMV class I MHC homolog, UL142, which is present in clinical HCMV strains such as Toledo[4]. UL142 shares about 20% amino acid sequence identity with MHC class I molecules. It could play a role in the virulence of the wild-type HCMV. Immunological, biochemical and structural studies are underway to characterize this protein. Altogether, our work on UL18 and UL142 will expand our basic understanding of the MHC class I homologs and the virus-host interaction.

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209. Structural studies of the interaction between V2R pheromone receptors and M10 MHC molecules

Rich Olson, *Kathryn E. Huey Tubman*

Major histocompatibility complex (MHC) molecules have been implicated in a number of non-immune roles in the central nervous system, particularly in synaptic development and plasticity. The discovery of M10 (50% sequence identity to classical MHC molecules) proteins expressed in the vomeronasal organ adds to the list of non-traditional roles of MHC homologs. M10 molecules associate with the V2R class of vomeronasal receptors, a family of G-protein coupled receptors (GPCRs) thought to function as pheromone receptors. Recent studies showing that classical MHC-binding peptides activate V2R-expressing neurons offer tempting clues that M10s might participate directly in the recognition of pheromone ligands, but M10 proteins do not bind to these peptides with significant affinity. Instead of presenting MHC-binding peptides, M10s might function as molecular chaperones to V2R receptors or more generally as modulators of neuronal function, as demonstrated elsewhere in the brain for classical MHC molecules.

We are using GFP-fusion constructs of V2R receptors to investigate expression strategies for making V2R/M10 complexes for structural studies. This includes using a fluorescence-detection size exclusion chromatography (FSEC) system to evaluate the chromatographic behavior of multiple genes in various detergents without the need for initial purification. Eventually we plan to scale up the expression of promising V2R/M10 complexes for S-ray crystallographic trials and electron microscopy. This information will allow us to uncover the unique nature of the interaction and design

functional experiments targeted towards understanding the possible role of MHC molecules in pheromone detection.

210. Improved neutralizing antibodies against HIV

Anthony P. West, Jr., Joshua Klein, Krystine Spiess, Priyanthi Peiris, Lili Yang, David Baltimore[*]

Together with David Baltimore's laboratory, we have begun an "Engineering Immunity" project that has the overall goal of developing a new approach to treating HIV/AIDS. This strategy envisions using lentiviral vectors to program a patient's immune system to produce designed anti-HIV molecules. The Bjorkman lab component of this project entails developing an improved set of antibody reagents that neutralize HIV. Although the vast majority of natural anti-HIV antibodies are highly strain-specific, a small number of broadly neutralizing anti-HIV antibodies have been identified. We are attempting to take this antibodies as a starting point to develop antibodies or antibody-like proteins engineered to bind more tightly to HIV or to recruit immune effector cells. The goal of our part of the Engineering Immunity project is to design, produce, and evaluate a series of optimized antibody or antibody-like anti-HIV proteins for further testing in a mouse model system.

[*]*President, Caltech*

211. Studies in the design and characterization of HIV neutralizing molecules

Joshua S. Klein

Human Immunodeficiency Virus (HIV) is estimated to currently infect approximately 40 million people worldwide and approximately three million AIDS-related deaths occurred in 2005. Conventional vaccine design methodologies have yet to yield an immunogen capable of eliciting protective immunity to HIV and the antiretroviral therapies credited with the precipitous decline in AIDS-related deaths in the developed world remain largely unavailable in underdeveloped regions of the world such as Southern Africa and parts of Asia, where most HIV infections occur. To address these issues, we are collaborating with the laboratory of David Baltimore to develop a hematopoietic stem cell transduction-based therapeutic vaccine capable of providing protective immunity and clearing an established infection. We have successfully produced a small array of single-chain Fv (scFv) anti-HIV molecules and shown them to be stable, monodispersed, able to bind their epitopes with low nanomolar affinities, and capable of neutralizing HIV pseudovirions *in vitro*. We are now in the process of fusing these molecules to various protein scaffolds in order to increase their potential therapeutic efficacies. For example, it has been estimated that peak HIV production in an infected individual may reach ten billion virions per day with at least one mutation in every virion produced, making the capacity to remove infected cells critical to reducing viral load and limiting the opportunity of escape by mutation. To address this issue, we will fuse our scFv molecules to an IgG-derived Fc domain bearing mutations recently observed to confer a much greater capacity for the recruitment of antibody-dependent cellular cytotoxicity, an important immune mechanism for clearing infected cells *in vivo*. We are also developing a novel method to examine the question of whether affinity correlates with neutralization effectiveness through the combination of surface plasmon resonance and multiplex array technologies. If a strong correlative relationship exists, this method would vastly increase the rate by which the effectiveness of constructs such as ours and other conventional antibodies may be assayed against meaningful numbers of different HIV isolates.

212. Structural study of L1 homophilic adhesion by cryoelectron tomography

Yongning He, Grant Jensen[*]

L1 (CD171) is a cell surface molecule expressed on many cell types, especially on nerve tissues, and involved in many cellular processes such as cell migration, axonal growth, myelination, etc. L1 mediates both homophilic and heterophilic adhesion for cells. The homophilic adhesion of L1 is important for cell migration for both neural and tumor cells, which plays an important role in tumor invasion and migration. L1 is transmembrane protein with a large extracellular portion, a single-pass transmembrane domain and a cytoplasm tail. The extracellular part contains six immunoglobulin (Ig) domains and five fibronectin type III (FN) domains. It has been shown that only ectodomains are required for performing homophilic cell adhesion function. Based on the known structure of L1 homologues, the four N-terminal Ig domains are hypothesized to adopt a horseshoe-shaped conformation in which the first and the second Ig domain folds back to interact with the fourth and the third Ig domains, respectively. The role of fibronectin domains in adhesion remains unclear. We are investigating L1-mediated homophilic adhesion using cryoelectron tomography (cryoET), a method to generate three-dimensional (3D) images of large molecular structures at resolutions approaching 4nm. Instead of using real cells for aggregation, we utilize synthetic liposomes to which the ectodomain of L1 has been attached. The L1 liposomes can be used to monitor homophilic adhesion and visualized directly, hence providing a 3D picture of homophilic adhesion. 3D tomograms of L1 show recognizable features at liposome surfaces and a common distance between adjacent adhering membranes. Furthermore, L1 forms regular structures between neighboring L1 molecules on adjacent membrane surfaces. Based on the observed structure features, a potential homophilic adhesion model for L1 has been proposed.

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213. Mono- and multivalent Fvs bind unaggregated poly-Gln according to linear lattice model

Pingwei Li, Kathryn E. Huey Tubman, Anthony P. West, Jr., Melanie J. Bennett

Huntington's and related neurological diseases are caused by the expansion of a CAG repeat encoding a poly-glutamine (poly-Gln) tract. Some antibodies preferentially recognize expanded poly-Gln (>36), which has been interpreted as supporting a model in which expanded poly-Gln adopts a pathologic conformation that can be targeted for drug design. However, our previous results demonstrated that huntingtin Exon 1 fusion proteins with 16 to 46 glutamines have random coil conformations in solution, with no evidence for a global conformational change above 36 glutamines, and that both normal and expanded poly-Gln bind anti-poly-Gln antibodies [1]. These results suggested that poly-Gln in the soluble (pre-aggregation) state is better described by a "linear lattice" model. In this model, soluble expanded poly-Gln differs from normal poly-Gln mainly in containing greater numbers of an epitope that is recognized by antibodies, rather than adopting a novel global fold. The linear lattice model predicts that multivalent compounds will bind with high avidity and specificity to expanded poly-Gln tracts. We expressed the Fv (variable domains) of the anti-poly-Gln antibody MW1, solved its crystal structure at 2.1Å, and produced monovalent and multivalent Fvs to compare their binding to normal and expanded poly-Gln. As predicted by the linear lattice model, dimeric and tetrameric anti-poly-Gln Fvs bind more tightly to long than to short poly-Gln tracts, and the multimeric Fvs bind expanded poly-Gln with higher apparent affinity than does the monomeric MW1 Fv. These results suggest that linkage of monovalent anti-poly-Gln compounds is an effective strategy to achieve therapeutics with high avidity and specificity for expanded poly-Gln in the pre-aggregation state.

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Professor Emeritus: Charles J. Brokaw

Summary: Motor enzymes — dyneins, kinesins, and myosins — convert energy from ATP dephosphorylation into most of the movements performed by eukaryotic cells. We think that myosin and kinesin are reasonably well understood, although new experimental results from time to time surprise us. On the other hand, we have very little knowledge or understanding of the functioning of the axonemal dyneins that power the movements of flagella and cilia; these molecular complexes are a major challenge for the future. My current work uses computer simulation methods to explore ideas about motor enzyme function in situations ranging from experimental studies on individual motors to an intact flagellum containing tens of thousands of dyneins. Some of the simulation programs, as Macintosh applications, are available at www.cco.caltech.edu/~brokawc/software.html

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Professor of Chemistry and Biology: Judith L. Campbell
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Summary: A hallmark of cancer cells, in addition to uncontrolled proliferation, is genomic instability, which appears in the form of chromosome loss or gain, gross chromosomal rearrangements, deletions, or amplifications. The mechanisms that suppress such instability are of the utmost interest in understanding the pathogenesis of cancer. Our lab studies the components of the DNA replication apparatus that promote genomic stability, primarily using yeast genetics, biochemistry, and functional genomics.

Several years ago, Rajiv Dua in the laboratory discovered that DNA polymerase ε, one of four essential DNA polymerases in yeast, had not one, but two essential functions. Deletion of the polymerase domain left the cells viable because another polymerase activity could substitute. Conversely, deletion of the remaining, non-catalytic half of the protein was lethal. Shaune Edwards in the laboratory carried out a two-hybrid screen for proteins that interact with the enigmatic C-terminal region of pol ε in order to discover its function. She found that pol ε interacts with Trf5, a protein involved in establishing cohesion of sister chromatids during passage of the replication fork. She has gone on to develop evidence that the essential function of the C terminus of pol ε is to aid in establishing efficient sister chromatid cohesion during S phase. Another postdoctoral fellow in the laboratory, Caroline Li has characterized the Trf5 protein. She has shown that it encodes a previously unknown poly A polymerase and that it stimulates the activity of pol ε dramatically. Future studies are aimed at defining the mechanism by which these two proteins regulate interaction of the replisome with the cohesin complex, the glue that holds the chromosomes together, and how failure of cohesion leads to genomic instability.

At least seven human diseases characterized by cancer predisposition and/or premature aging are correlated with defects in genes encoding DNA helicases. The yeast genome contains 134 open reading frames with helicase motifs, only eight of which have been characterized. Martin Budd in our laboratory identified the first eukaryotic helicase essential for DNA replication, Dna2. He showed by interaction studies that it was a component of the machine that is required for accurate processing of Okazaki fragments during lagging strand DNA replication. Enzymatic studies to elucidate the sequential action of the DNA polymerase, helicase, and nuclease required for this processing form an ongoing mechanistic biochemistry project in the laboratory.

Stimulated by various reports in the literature implicating Dna2 in telomere biogenesis and structure, Wonchae Choe made the interesting observation that the bulk of Dna2 is localized to telomeres and that this localization is dynamic. During G1 and G2 phases of the cell cycle, Dna2 is at telomeres. During S phase Dna2 is present on the replicating chromatin. Current studies are aimed at defining the genes that regulate the localization, including phosphorylation by the yeast ATR ortholog, Mec1. In addition to defects in replication, *dna2* mutants are also very sensitive to agents that induce double strand breaks (DSBs). Osamu Imamura has shown that Dna2 is mobilized from telomeres in response to the induction of double strand breaks. He is carrying out experiments to test the model that Dna2 delocalization from telomeres is part of the signaling system that induces the DNA damage and S phase checkpoints, as has also been suggested for yKU, a protein involved in non-homologous end joining and in stabilizing telomeres.

One model of cellular aging suggests that accumulation of DNA damage leads to replicative senescence. Most endogenous damage occurs during S phase and leads to replication fork stress. At least three human diseases of premature aging or cancer predisposition, Werner, Bloom, and Rothmund-Thompson, are caused by defects in helicases similar to Dna2. Martin Budd and Laura Hoopes found that *dna2* mutants have a significantly reduced life span. Microarray analysis by Isabelle Lesur shows that the *dna2* mutants age by the same pathway as wild-type cells; they just age faster. Interestingly, the human Bloom and Werner genes suppress the replication defect of *dna2* mutants. Yeast transcriptome analysis shows that old *dna2* mutants have a gene expression pattern strikingly similar to cells senescing due to telomerase defects. Future work will take advantage of the yeast system to further delineate the role of BLM and WRN proteins in mammalian cells. The work of Tao Wei in the lab suggests that instability of repetitive DNA, such as the ribosomal locus and telomeric DNA, is a major cause of genomic instability in the aging *dna2* mutants.

214. Characterization of *ftsZ*-like sequences in *Magnetospirillum magnetotacticum*

Maria K.L. Ho[1], *Elizabeth Bertani*

The *fts*Z gene encodes an analogue of tubulin and is essential for bacterial cell division. Although most organisms have just one such gene, four sequences with a tubulin signature can be seen in the draft genome sequence of the magnetotactic organism, *Magnetospirillum magnetotacticum.* The putative proteins have identities of 53% (1), 52% (2), 49% (3) and 42% (4) to *E. coli ftsZ.* Paralogs 1-3 have the conserved "synergy loop," necessary for GTP hydrolysis, that is found in all tubulins, whereas the loop in paralog 4 is incomplete. Paralog 1 is clustered together with other genes involved in cell division, but the

other three are scattered at different sites in the chromosome.

What are the functions of the four *Magnetospirillum* paralogs? Because of its conserved structure and location, paralog 1 seems most likely to be the *fts*Z gene responsible for cell division in *Magnetospirillum.* The others might be involved in the division or organization of the magnetite-containing organelle that makes the bacteria magnetotactic, although multiple copies of *fts*Z are not necessarily found in other magnetotactic organisms.

If a heterologous *fts*Z gene is expressed in *E. coli*, cell division is blocked and the bacteria form long filaments. When the paralog 2 protein is overexpressed in *E. coli*, the bacteria form long filaments and are unable to produce colonies. Thus, in spite of the conservation seen in paralog 1, paralog 2 has the phenotype expected of a true ftsZ gene. In contrast, overexpression of the paralog 4 protein does not result in filament formation and does not affect colony formation. The phenotypes of the other two paralogs remain to be investigated.

Are all these paralogs expressed in *Magnetospirillum*? In *E. coli* the ftsZ protein is found scattered throughout the cytoplasm, until cell division when it localizes to the midplate. We will attempt to detect the presence of the proteins in *Magnetospirillum* by Western blotting using antibody against *E. coli* ftsZ.

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215. Strand annealing and strand exchange activities of Dna2

Taro Masuda Sasa, Piotr J. J. Polaczek

DNA2 is an essential gene conserved from yeast to human. Budding yeast Dna2p (ScDna2p) is a nuclease/helicase, and required for assisting FEN1 nuclease in processing a subset of Okazaki fragments that have long, single-stranded 5' flaps. Additionally, *DNA2* performs an essential function in the maintenance of telomeres. In *S. cerevisiae*, *dna2* mutants have defects in the maintenance of telomeres and in *de novo* telomere synthesis, and ScDna2p shows dynamic localization to telomeres at G1 and G2 phases.

Last year, I have characterized biochemical activity of human Dna2 (hDna2p), showing that both of nuclease and helicase activities of Dna2p are conserved in eukaryotes [1]. Further studies in this year demonstrated two novel activities of both the yeast and human Dna2 helicase/nuclease protein: ATP independent single-strand annealing and strand exchange. These activities are independent of ATPase/helicase and nuclease activities in that mutations eliminating either nuclease or ATPase/helicase do not inhibit strand annealing or strand exchange. Since similar annealing/exchange properties have been reported for BLM and WRN, which has been shown to suppress yeast dna2 mutant, we propose a model that the shared strand annealing/exchange activities of Dna2, BLM, and WRN affect the kinetics of flap equilibration, promoting the processing of Okazaki fragment flaps during DNA replication and repair [2]. Another interesting property of yeast DNA2 is its interaction with PIF1, encoding a helicase whose major function is to regulate telomere length by inhibition of telomerase. A deletion of DNA2 is normally lethal, but the lethality can be suppressed by deletion of PIF1. To investigate the interaction between DNA2 and PIF1 biochemically, we investigated if nuclease of Dna2 is stimulated by Pif1 protein. Preliminary results show the efficient cleavage of short fork substrates only in the presence of both Pif1 and Dna2. This stimulation was dependent on ATP. On the basis of these results, we suggest that the helicase activity of Pif1 provides a substrate for Dna2 nuclease, explaining why deletion of PIF1 can suppress dna2 null mutant.

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216. Synthetic lethal and synthetic suppressive interactions with dna2 mutants

Martin Budd, Amy Tong[*]*, Charles Boone*[*]*, Judith L. Campbell*

To elucidate the network that maintains high fidelity replication, we have introduced two mutant alleles of Dna2 into each of 4700 viable yeast deletion mutants and determined the fitness of the double mutants. Fifty-six Dna2 interacting genes were identified. Clustering analysis of genomic synthetic lethality profiles of each of the 43 of the Dna2 interacting genes defines a network consisting of 322 genes and 876 interactions whose topology provides clues as to how replication proteins coordinate replication and repair to protect genome integrity. In this network, Dna2 is one of the major hubs that link DNA replication, DNA repair, cell cycle checkpoints, chromatin structure, osmotic stress, oxidative stress, and RNA metabolism.

From work on the network, an important interaction of Dna2 with Pif1 was analyzed. Pif1 is a 5' to 3' helicase whose major function is regulation of telomere length and mitochondrial recombination. The lethality of dna2D can be suppressed by deletion of Pif1. The *dna2*Δ *pif1*Δ strain is temperature sensitive and mms sensitive. The temperature sensitivity of the *dna2*Δ *pif1*Δ strain is suppressed by an additional deletion of Pol32, and subunit of DNA polymerase . Deletion of Pif1 presumably results in a reduction of long flaps requiring Dna2 for processing. In addition, Pif1 has synthetic suppressive and sick interactions with pol32, rad27, and pol1 mutants demonstrating a multiprotein interaction of pif1 with lagging-strand proteins.

In addition we are examining the role of Dna2 in the synthesis of telomeres. Dna2 is required for creating the long telomeres in the pif1 mutant. Dna2 and Exo1 nucleases share redundant roles in OFP at the telomeres

since dna2-2 exo1 mutants have increased amounts of single-stranded DNA at the telomere overhang. We are analyzing the overlapping roles of Dna2 and Mre11 in synthesizing the telomere overhang.

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217. Physical Interaction between *Saccharomyces cerevisiae* DNA Pol epsilon and Mrc1

Huiqiang Lou, Clara Reis, Martin Budd, Katsuhiko Shirahige, Judith L. Campbell*

The only essential function of Pol epsilon, which lies at the extreme C-terminus of Pol2, still remains enigmatic. Our finding of the *in vivo* interaction between Mrc1 and Pol2 might shed light on this puzzle, because, like Pol2, Mrc1 participates in DNA replication as part of the replication fork, the cell cycle checkpoint as a key mediator, and sister chromatid cohesion.

The interaction between Mrc1 and Pol2 was firstly identified in a global two-hybrid screen for all gene products in yeast that interact specifically with the Pol2 C terminus. Both Mrc1 and the non-phosphorylatable *mrc1AQ* mutant interact with Pol2-C terminus. It suggests that this interaction is independent of phosphorylation. We characterized the interactions functionally using yeast genetic techniques - synthetic lethality and suppression. The *mrc1Δ* and *pol2 11* double mutant strain is lethal, while the *mrc1AQ* and *pol2 11* double mutant stain is viable. The *mrc1AQ* mutant is defective in the checkpoint but not in DNA replication. Furthermore, the latter double mutant strain shows almost the same sensitivity to growth temperature and drugs (HU, MMS) as a *pol2 11* mutant strain. The *mrc1AQ/pol2 11/rad9Δ* triple mutant strain is viable, but very sick. In response to DNA replication stress, Mrc1 and Pol2 may probably be involved in the same checkpoint pathway, that is, they are epistatic in the checkpoint pathway, while Rad9 belongs to another parallel pathway. Strains transformed with the multicopy plasmid containing the *MRC1* gene can grow at temperatures higher than those transformed with the empty vector. Overexpression of *MRC1* can also suppress HU sensitivity of the *pol2 11* cells. This suggests that overproduction of Mrc1 can help mutant *pol2 11* protein folding, which in turn may mean that they truly interact physically *in vivo*.

Interaction between Mrc1 and Pol2 and their interaction domain are characterized by the *in vivo* coimmunoprecipitation assay. Mrc1 partially coimmunoprecipitates with Pol2, while almost all Pol2 molecules precipitate with Mrc1 in asynchronized cells. This also suggests that Pol2 associates with Mrc1 throughout the whole cell cycle, which is further confirmed by using α–factor arrested (G1) and HU-arrested (S) cells. HU-treated cells also show that both phosphorylated and nonphosphorylated Mrc1 bind to Pol2 with similar efficiency. The interaction remains undisturbed with up to 250 mM salt. Through a set of N- or C-terminal truncation mutants of Mrc1, N-terminus (140-400aa) of Mrc1 seems to be responsible for binding with Pol2. Interaction domain of Pol2 is currently under investigation.

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218. Contribution of Trf4/5 and the nuclear exosome to genome stability through regulation of histone mRNA levels in *Saccharomyces cerevisiae*

Clara C. Reis, Judith L. Campbell

Balanced levels of histones are crucial for chromosome stability, and one major component of this control regulates histone mRNA amounts. The *Saccharomyces cerevisiae* poly(A) polymerases Trf4 and Trf5 are involved in a quality control mechanism that mediates polyadenylation and consequent degradation of various RNA species by the nuclear exosome. None of the known RNA targets, however, explains the fact that *trf* mutants have specific cell cycle defects consistent with a role in maintaining genome stability. Here, we investigate the role of Trf4/5 in regulation of histone mRNA levels. We show that loss of Trf4 and Trf5, or of Rrp6, a component of the nuclear exosome, results in elevated levels of transcripts encoding DNA replication-dependent histones. Suggesting that increased histone levels account for the phenotypes of *trf* mutants, we find that *TRF4* shows synthetic genetic interactions with genes that negatively regulate histone levels, including *RAD53*. Moreover, synthetic lethality of *trf4Δ rad53Δ* is rescued by reducing histone levels whereas overproduction of histones is deleterious to *trfs* and *rrp6Δ* mutants. These results identify *TRF4*, *TRF5,* and *RRP6* as new players in the regulation of histone mRNA levels in yeast. To our knowledge, the histone transcripts are the first mRNAs that are upregulated in Trf mutants.

219. Dna2 helicase/nuclease is a substrate of Mec1

Barbara Fortini

The Dna2 protein is a yeast helicase/nuclease that is involved in Okazaki fragment processing, repair of X-ray- and MMS-induced DNA damage, rDNA stability, and the telomere position effect. We have shown previously that Dna2 exhibits an unusual subcellular distribution for a replication protein. The bulk of Dna2 is localized to telomeres in G1, leaves telomeres in S phase to be found at internal chromosomal sites, and returns to telomeres in G2. In addition, upon treatment with DNA damaging agents, Dna2 is released from telomeres. We have also shown that damage-induced release from

telomeres depends on the Mec1 checkpoint kinase. Further, an *in vitro* kinase assay demonstrates that Dna2 is an efficient substrate of purified Mec1 kinase. Mec1 is member of the PI-3-kinase-related kinase (PIKK) family whose members preferentially phosphorylate SQ or TQ motifs upon DNA damage. To ascertain whether Dna2 is an *in vivo* substrate of Mec1, we have used site-directed mutagenesis to alter the putative Mec1 phosphorylation sites in Dna2 to alanine residues and analyzed the phenotypes of the resulting mutants. Two of these single mutants show increased DNA damage sensitivity to a variety of DNA damaging and replication stress causing agents. Chromatin immunoprecipitation experiments also show altered telomere-Dna2 localization patterns for these SQ mutants, suggesting Mec1 phosphorylation may play a role in regulating the function of Dna2.

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Summary: The primary focus of our lab is to understand the role of mitochondrial dynamics in normal cellular function and human disease. Mitochondria are remarkably dynamic organelles that undergo continual cycles of fusion and fission. The equilibrium of these two opposing processes determines the overall morphology of mitochondria in cells and has important consequences for mitochondrial function.

Our research falls into several broad areas:
(1) What are the cellular and physiological functions of mitochondrial fusion and fission?
(2) What is the molecular mechanism of mitochondrial membrane fusion and fission?
(3) What role do mitochondrial dynamics play in human diseases?

To address these issues, we use a wide range of approaches, including genetics, biochemistry, cell biology, and structural biology.

Cellular and physiological functions of mitochondrial fusion and fission

A typical mammalian cell can have hundreds of mitochondria. However, each mitochondrion is not autonomous, because fusion and fission events mix mitochondrial membranes and contents. As a result, such events have major implications for the function of the mitochondrial population. We are interested in understanding the cellular role of mitochondrial dynamics, and how changes in mitochondrial dynamics can affect the function of vertebrate tissues.

Much of our work focuses on proteins called mitofusins (Mfn1 and Mfn2), which are transmembrane GTPases embedded in the outer membrane of mitochondria. These proteins are essential for fusion of mitochondria. To understand the role of mitochondrial fusion in vertebrates, we have constructed mice deficient in either Mfn1 or Mfn2. We find that mice deficient in either Mfn1 or Mfn2 die in mid-gestation. Mfn2 mutant embryos have a specific and severe disruption of a layer of the placenta called the trophoblast giant cell layer. These findings indicate that mitochondrial fusion is essential for embryonic development due to a cell type-specific dependence on mitochondrial fusion. More recently, we have generated mice with conditional alleles of Mfn1 and Mfn2, and analyses of these mice indicate important roles for mitochondrial fusion in adult tissues such as brain and skeletal muscle (Hsiuchen Chen). These studies are relevant to our understanding of several human diseases, including mitochondrial encephalomyopathies and peripheral nerve degeneration.

Embryonic fibroblasts lacking Mfn1 or Mfn2 display fragmented mitochondria, a phenotype due to a severe reduction in mitochondrial fusion. Cells lacking both Mfn1 and Mfn2 have completely fragmented mitochondria and show no detectable mitochondrial fusion activity. Our analysis indicates that mitochondrial fusion is important not only for maintenance of mitochondrial morphology, but also for cell growth, mitochondrial membrane potential, and respiration (Hsiuchen Chen). We are also using RNA interference to disrupt the function of other proteins involved in mitochondrial fusion and fission.

Molecular mechanism of membrane fusion and fission

The best understood membrane fusion proteins are viral envelope proteins and SNARE complexes. Viral envelope proteins, such as gp41 of HIV, reside on the lipid surface of viruses and mediate fusion between the viral and cellular membranes during virus entry. SNARE complexes mediate a wide range of membrane fusion events between cellular membranes. In both cases, cellular and crystallographic studies have shown that the formation of helical bundles plays a critical role in bringing the merging membrane together. We would like to understand mitochondrial fusion at a similar level of resolution and to determine whether there are common features to these diverse forms of membrane fusion.

Mitofusins are the only conserved mitochondrial outer membrane proteins involved in fusion. Therefore, it is likely that they directly mediate membrane fusion. Consistent with this idea, mitofusins are required on adjacent mitochondria to mediate fusion. In addition, mitofusins form homotypic and heterotypic complexes that are capable of tethering mitochondria. We are trying to determine how tethered mitochondria, mediated by mitofusins, proceeds to full fusion. It should be noted that mitochondrial fusion is likely to be more complicated than most other intracellular membrane fusion events, because four lipid bilayers must be coordinately fused. Our work with mammalian cells carrying mutant Mfn2 molecules indicates that Mfn1 and Mfn2 cooperate closely with each other to mediate fusion (Scott Detmer). In addition to studies in mammalian cells, we have used yeast studies to show that mitofusins engage in key intermolecular interactions necessary for fusion (Erik Griffin).

We have used proteomic approaches in yeast cells to identify novel proteins involved in mitochondrial fusion and fission (Erik Griffin). The structures of proteins involved in mitochondrial fusion and fission are being solved by X-ray crystallography to gain an understanding of molecular mechanism. For example, we have recently

solved the structure of Fis1 in complex with a fragment of Mdv1 (Yan Zhang). This structure provides a basis for understanding assembly of the mitochondrial fission apparatus.

Mitochondrial dynamics in human disease

Two inherited human diseases are caused by defects in mitochondrial dynamics. Charcot-Marie-Tooth (CMT) disease is a neurological disorder that affects the peripheral nerves. Patients with CMT experience progressive weakness of the distal limbs and some loss of sensation. A specific type of CMT, termed CMT2A, is caused by mutations in Mfn2 and result from degeneration of axons in peripheral nerves. We are currently analyzing the functional consequences of such disease alleles, and using transgenic and targeted mutagenesis approaches to develop mouse models (Scott Detmer).

The most common inherited form of optic neuropathy (autosomal dominant optic atrophy) is caused by mutations in OPA1. This mitochondrial protein is localized to the intermembrane space and is essential for mitochondrial fusion. Little is known about the mechanism of OPA1 action. In particular, there has been no good cell culture system to perform basic structure-function analysis of OPA1. To develop such a system, we have collaborated with Christiane Alexander (Max-Delbrück-Center for Molecular Medicine) to isolate mouse cell lines lacking OPA1 (Hsiuchen Chen). Using these cell lines, we have identified a protein that regulates the activity of OPA1 and thereby the levels of mitochondrial fusion (Zhiyin Song).

Finally, an understanding of mitochondrial dynamics will be essential for understanding a large collection of diseases termed mitochondrial encephalomyopathies. Many mitochondrial encephalomyopathies result from mutations in mitochondrial DNA (mtDNA). In mtDNA diseases, tissues maintain their mitochondrial function until pathogenic mtDNA levels exceed a critical threshold. Experiments with cell hybrids indicate that mitochondrial fusion, by enabling cooperation between mitochondria, can protect respiration even when >50% of mtDNAs are mutant. To understand the pathogenesis of mtDNA diseases, it is critical to explore how mitochondria can be functionally distinct and yet cooperate as a population within a cell. We anticipate that our studies with mice lacking mitochondrial fusion will help to shed light on this group of often devastating diseases (Hsiuchen Chen).

220. Mitochondrial fusion is required for brain development

Hsiuchen Chen

Mitochondrial fusion is developmentally and physiologically important. Genetic targeting of any of the three known fusion genes, Mfn1, Mfn2, and OPA1, leads to embryonic lethality. Underscoring the importance of mitochondrial fusion in humans, two neurodegenerative diseases, Charcot-Marie-Tooth disease type 2A (CMT2A) and autosomal dominant optic atrophy (DOA), are caused by mutations in Mfn2 and OPA1, respectively.

To further explore the physiological requirements for mitochondrial fusion, we have created mice carrying conditional null alleles for both Mfn1 and Mfn2. Analysis of these mice has revealed several tissues particularly dependent on mitochondrial fusion. First, Mfn2 is required for proper cerebellar development during the postnatal period. When Mfn2 is disrupted in cerebellar primordia during embryogenesis, young pups display severe problems in balance and motility, resulting in feeding problems and death by about two weeks of age. Histologically, the mutant cerebella are much smaller than wild-type cerebella due to apoptotic cell loss. We have traced the primary defect in these animals to improper development of the cerebellar Purkinje cells. When Mfn2 is disrupted in mature Purkinje cells (the only efferent processes of the cerebellum), electron transport activity is affected and the neurons degenerate. As a result, the mice develop a resting tremor and have poor coordination while walking, classic signs of cerebellar dysfunction. Thus, mitochondrial fusion is required in both the development of cerebellar Purkinje cells as well as their maintenance. Our studies provide a powerful model system to study the role of mitochondrial fusion in neurodegenerative disease, with relevance to human disorders. In addition, they show that mitochondrial fusion is required in the central nervous system as well as in the peripheral motor neurons and optic nerve affected in CMT and DOA.

2221. Mitochondrial fusion and muscle function

Hsiuchen Chen

Many mitochondrial encephalomyopathies are caused by mutations in mitochondrial DNA (mtDNA). Although many disease-related mtDNA mutations have been documented, the pathogenesis of these diseases remains incompletely understood. In particular, little is known about the natural history of mtDNA mutations, and why certain cells accumulate high levels of mutant mtDNA. Mitochondrial fusion is likely to play an important role in modulating the inheritance of mutant mtDNA molecules during cell division, because fusion mixes contents between mitochondria, including mtDNA.

To explore these issues, we have disrupted both Mfn1 and Mfn2 in skeletal muscle. Such mutant mice are severely runted and die at around two months of age. Muscle mass is greatly reduced in these mice, and histological analysis reveals significantly decreased muscle fiber diameters. In addition, a succinate-dehydrogenase (SDH) enzymatic assay reveals greatly increased activity in all fibers. Increased SDH activity is a sign of mitochondrial proliferation and is a hallmark of many mitochondrial diseases. Our results suggest a common link between deficiencies in mitochondrial fusion and traditional mitochondrial diseases.

222. Functional studies of Mfn2 alleles found in Charcot-Marie-Tooth disease

Scott Detmer

Mitochondrial undergo continual fusion and fission, and these dynamic properties are important for mitochondrial and cellular function. Interestingly, mutations in two of the known mammalian mitochondrial fusion proteins, Mfn2 and OPA1, cause neurodegenerative disease in humans. In the case of Mfn2, over 40 mutations have been reported that cause Charcot-Marie-Tooth disease Type2A (CMT2A), a peripheral motor neuropathy that results in distal muscle and sensory deficits. We are using cell culture and biochemical experiments to functionally study these disease alleles.

We have characterized the nine originally reported Mfn2 CMT2A alleles. We find that all these alleles localize normally to mitochondria when expressed in mouse fibroblasts. Like wild-type Mfn2, the majority of these alleles co-immunoprecipitate with both Mfn1 and Mfn2. However, we find that the CMT2A alleles are functionally heterogeneous - approximately half can promote mitochondrial fusion and tubulation in double Mfn-null cells whereas the remaining cannot. In addition, when overexpressed, both the functional and non-functional alleles induce dramatic mitochondrial aggregation that is not observed with wild-type Mfn2. These phenotypes likely represent a conformational change in the disease alleles and may be related to disease progression.

Mfn1 and Mfn2 can cooperate in promoting mitochondrial fusion. We further find that the non-functional Mfn2 CMT2A alleles can be complemented by wild-type Mfn1, but not wild-type Mfn2, to form fusion active complexes. That is, the non-functional Mfn2 CMT2A alleles can promote tubulation in the presence of Mfn1 but not in the presence of Mfn2. These results provide a testable hypothesis for the specific loss of motor and sensory axons in CMT disease. Mfn1 appears to have a protective role in masking the defect in some CMT2A alleles, and the relative expression level of Mfn1 and Mfn2 in specific tissues may be the key to the cell type specificity.

223. Mouse models of Charcot-Maria-Tooth Disease Type 2A

Scott Detmer

We have established both transgenic and knock-in mouse models of CMT2A. We have generated multiple transgenic lines of mice expressing mutant Mfn2 under a motor neuron-specific promoter and have confirmed localized transgene expression. Interestingly, one of these lines exhibits a striking phenotype: affected mice are unable to dorsi-flex their hindpaws and subsequently display a gait defect characterized by dragging of the hindlimbs during walking. Over time this leads to deformities in the hindlimbs. This phenotype is remarkably similar to the symptoms of CMT2A patients. Such patients have distal limb weakness, which is manifested by an inability to dorsi-flex the foot, leading to "foot drop" during walking. Eventually this leads to foot deformities such as hammertoe caused by muscle imbalance.

In a second approach, we have generated knock-in mutations corresponding to two of the Mfn2 mutant alleles. Transmission and expression of the knock-in alleles have been confirmed. In the heterozygous state neither allele manifests a behavioral phenotype up to six months of age; we will continue to age and monitor these animals. However, for one of the alleles, both homozygous mutant animals and animals with the mutant allele over a Mfn2 null allele develop an unsteady gait, are severely runted and die by four weeks of age. By pinpointing the physiological defects in these animals, we will also gain clues to more subtle defects in the heterozygous animals. We are characterizing both transgenic and knock-in mice for motor neuron function, fitness, neuronal histology, and neuronal mitochondrial morphology. We expect these to be valuable models for the study of CMT disease progression.

224. Structural and functional studies of the fission apparatus of mitochondria

Yan Zhang

Mitochondrial fission in *Saccharomyces cerevisiae* is mediated by Dnm1p, Fis1p, Mdv1p, and Caf4p. Dnm1p is a dynamin-related GTPase that is mainly localized to punctate mitochondrial structures, a subset of which proceed to mitochondrial fission. The formation of normal Dnm1p puncta requires the mitochondrial outer membrane anchor protein Fis1p. The core domain of Fis1 consists of a six α-helical bundle composed of two tetratricopeptide repeats (TPR). Previous studies in our lab indicated that Fis1p mediates the mitochondrial localization of Dnm1p through its interactions with two adaptor proteins, Mdv1p or Caf4p. Both Mdv1p and Caf4p can independently recruit Dnm1p, but complexes recruited by Mdv1p appear to be more active for fission.

To elucidate the mechanism of Dnm1p recruitment to mitochondria, we are studying the interactions between Fis1p and the adaptor proteins Mdv1p and Caf4p biochemically and structurally. We have established a system in *E. coli* to co-express the cytosolic portion of Fis1p with a His-tagged interacting fragment of Mdv1p or Caf4p. Purification of the His-tagged fragment leads to co-purification of Fis1p. The complexes of Fis1p/Mdv1p and Fis1p/Caf4p have been purified and crystallized. The structure of the Fis1p/Mdv1p complex has been solved by molecular replacement using the structure of Fis1p as a model. The structure reveals that a stretch of residues on Mdv1p forms an α helix that binds in a novel mode to the TPR domains on Fis1p. Mutational studies of critical Mdv1 residues confirm the *in vivo* relevance of this binding mode. Meanwhile, a native data set of the Fis1p/Caf4p complex has been collected but phasing using molecular replacement with the structure of Fis1p as a model has failed. Heavy metal derivatives are being screened for phasing. These structural studies will

be essential for understanding assembly of the mitochondrial fission apparatus.

225. Domain interactions within Fzo1 oligomers are essential for mitochondrial fusion

Erik Griffin

Mitofusins are conserved GTPases essential for the fusion of mitochondria. These mitochondrial outer membrane proteins contain a GTPase domain and two or three regions with hydrophobic heptad repeats, but little is known about how these domains interact to mediate mitochondrial fusion. To address this issue, we have analyzed the yeast mitofusin Fzo1p and find that mutation of any of the three-heptad repeat regions (HRN, HR1, and HR2) leads to a null allele. Specific pairs of null alleles show robust complementation, indicating that functional domains need not exist on the same molecule. Biochemical analysis indicates that this complementation is due to Fzo1p oligomerization mediated by multiple domain interactions. Moreover, we find that two non-overlapping protein fragments, one consisting of HRN/GTPase and the other consisting of HR1/HR2, can form a complex that reconstitutes Fzo1p fusion activity. Each of the null alleles disrupts the interaction of these two fragments, suggesting that we have identified a key interaction involving the GTPase domain and heptad repeats essential for fusion.

226. Regulation of mitochondrial fusion

Zhiyin Zhang

To identify molecules involved in control of mitochondrial fusion, we used tandem affinity purification of Mfn1 and Mfn2 from mouse cells to identify mitofusin-associated proteins by mass spectrometry. Such associated proteins were screened by RNAi knockdown to identify those involved in mitochondrial fusion. Remarkably, we found a protein that is essential both for mitochondrial fusion and also for regulating the proteolytic processing of OPA1. OPA1 is proteolytically processed to a long and short isoform. In yeast cells, both forms are necessary for mitochondrial fusion, but their functions in mammalian cells are poorly understood. We are currently using OPA1-null cells to explore how the processing of OPA1 is regulated to control its fusion activity.

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Summary: The Deshaies lab works on two basic biological processes: Control of cell division, and regulation of cell function by attachment of ubiquitin or ubiquitin-like proteins to target polypeptides. We are particularly interested in how attachment of ubiquitin to proteins enables their degradation, and how protein degradation via this mechanism is used to regulate cell division.

Defective control of cell division can result in disease, as when unrestrained cell proliferation leads to cancer. Defects of the ubiquitin system can also lead to cancer, as well as neurodegenerative diseases. An understanding of how the cell division machinery and the ubiquitin system operate will thus, provide insight into basic cellular processes essential to the life of eukaryotic cells, and may suggest cures for diseases that affect millions of people.

We are using biochemical, molecular, and genetic approaches in baker's yeast and mammalian cells to investigate cell proliferation and the ubiquitin system. Our long-term goal is to understand how these processes work and how they are controlled. Baker's yeast is an excellent organism for basic cell biological studies because it is easy to work with, and many studies have confirmed that yeast and animal cells largely use the same proteins to regulate basic cellular processes.

Below, I summarize in more detail the four major areas of investigation in the lab, and provide thumbnail descriptions of all current projects.

SCF ubiquitin ligases: Mechanism, regulation, and physiology

Cellular proteins are marked for degradation by attachment of the polypeptide ubiquitin. Ubiquitin is attached to substrates by a cascade comprising ubiquitin-activating (E1), ubiquitin-conjugating (E2), and ubiquitin ligase (E3) enzymes. Ubiquitination occurs when an E3 enzyme binds to both substrate and E2 ubiquitin conjugating enzyme, bringing them into proximity so that ubiquitin is transferred from the E2 to substrate. Specificity and regulation of ubiquitination are typically imparted by E3s, which are the most diverse components of the system. Once ubiquitin is attached to a substrate, the reaction can either terminate (in which case the ubiquitin serves as a regulatory signal to modulate protein function or localization) or continue, leading to the assembly of a multiubiquitin chain. A chain of four ubiquitins suffices to specify destruction of the substrate by the proteasome.

Mechanism of action of SCF ligases: In 1999, we and others reported that RING domains underlie ubiquitin ligase activity (Seol *et al*., 1999). This discovery revealed the largest class of ubiquitin ligases, with up to 385 members. The progenitor of the RING-based ubiquitin ligases, SCF (which we co-discovered in 1997) (Feldman *et al*., 1997), defines a subfamily of multisubunit cullin–RING ligases that may number as many as 300-350 members, due to combinatorial mixing of subunits. Thus, there may be as many as 700 distinct RING ligase complexes, which would make it the largest-known family of enzymes in human cells (Petroski and Deshaies, 2005). As befits such a large family, the cullin–RING ligases have been implicated in a dazzling array of cellular and organismal processes, ranging from circadian rhythms to sulfur assimilation. However, despite the biological import of these enzymes, the mechanism of how they work remains unknown.

Over the past few years, we have made substantial progress towards understanding how SCF enzymes work. Our key innovation was to develop a reconstituted system in which a physiological substrate (budding yeast Cdk inhibitor Sic1 assembled into cyclin–Cdk complexes) is ubiquitinated by SCF and degraded by the proteasome – work that was carried out by Renny Feldman, Craig Correll, and Rati Verma (Feldman *et al*., 1997; Verma *et al*., 2001). Matt Petroski then constructed Sic1 substrates bearing single ubiquitin acceptor lysines, and used these substrates to characterize the impact of ubiquitin chain position on substrate recognition and degradation by the proteasome (Petroski and Deshaies, 2003).

We believe that the system that we have developed is the best available for studying biochemical mechanisms of ubiquitination, because our substrate is multiubiquitinated and degraded at rates that approach those that must occur *in vivo*, and the nature of the reaction products is defined due to the substrate having only a single lysine acceptor. I now perceive a terrific opportunity to exploit our *in vitro* systems to address basic questions that are of central importance to understanding the mechanisms that underlie the operation of the ubiquitin-proteasome system (UPS). For example, how does a RING domain activate ubiquitin transfer from ubiquitin-conjugating enzyme to substrate? What is the basis for the synthesis of the lysine 48-linked ubiquitin chains that signal proteolysis? How is processive ubiquitination of Sic1 achieved, and how does it relate to the dynamics of substrate and Cdc34 (E2) association with SCF? How does ubiquitin transfer occur across an ~50 Å gap that is thought to exist between the substrate and E2 enzyme bound to SCF? None of these questions are

resolved for any RING E3, and thus, illuminating the answers will establish paradigms that inform our understanding of how hundreds of ubiquitin ligases work. The insights that emerge from this effort may also provide clues to the development of drugs that modulate the activity of RING-based ligases.

Regulation of SCF ubiquitin ligases: It was originally thought that SCF ubiquitin ligases are constitutively active, and substrate turnover is regulated by phosphorylation of the substrate. Subsequently, it was shown that the Cul1 subunit of SCF is modified covalently by the ubiquitin-like protein Nedd8, thus raising the possibility that SCF might be regulated post-translationally. In 2001, two students from the lab, Svetlana Lyapina and Greg Cope, reported that a poorly understood protein complex known as COP9 Signalosome (CSN) binds SCF in animal cells, and promotes the cleavage of Nedd8 from Cul1 via a tightly-associated Nedd8 isopeptidase activity (Lyapina *et al.,* 2001). This was the first biochemical function ascribed to CSN, and opened the door to the study of SCF regulation by reversible cycles of 'neddylation.' Subsequently, Greg Cope discovered that the Csn5 subunit harbors a motif that we named 'JAMM' (for JAb1/Mpn domain Metalloenzyme) (Cope *et al*., 2002). We predicted that JAMM comprises a novel metalloprotease active site. Later, Xavier Ambroggio, who was a joint student with Doug Rees in Chemistry, substantiated this prediction by employing X-ray crystallography to show that the conserved residues of the JAMM motif coordinate a zinc ion in an active site-like cleft of the protein AF2198 from *Archaeglobulus fulgidis* (Ambroggio *et al.,* 2004). We continue to investigate the regulation of SCF by CSN. We hope to understand how CSN itself is controlled, and what role CSN plays in sustaining active SCF complexes and sculpting the repertoire of SCF complexes in a cell.

Mechanism of action and regulation of the 26S proteasome

Once substrates are ubiquitinated by E3s, they are degraded by the 26S proteasome. The 26S proteasome is a large protein machine that comprises two major subcomplexes: The 20S 'core' proteasome and the 19S regulatory 'cap.' The 20S core forms a cylindrical structure that houses the protease active sites of the proteasome. Each end of the 20S cylinder is decorated by a 19S cap. The 19S cap can be further subdivided into the 'lid' and the 'base.' The base contains six ATPases that are thought to form a ring that abuts the end of the 20S cylinder. The lid, in turn, sits upon the base. The base is thought to control access of substrates into the 20S proteolytic chamber, whereas the lid confers ubiquitin-dependence. The 26S proteasome degrades proteins that are linked to a chain of at least four ubiquitins. The tetraubiquitin chain mediates binding of the attached substrate to the proteasome, after which it is disengaged from bound partners, unfolded, deubiquitinated, and translocated into the proteolytic chamber of the proteasome where the denuded substrate is degraded.

A fully reconstituted system to study Sic1 degradation: Pre-2000, all *in vitro* studies of protein degradation by the UPS relied either on crude systems such as *Xenopus* or reticulocyte extracts, or non-natural substrates. No assay was available to harness the power of yeast molecular genetics to enable dissection of the mechanism-of-action of the proteasome. We thus, developed a system wherein ubiquitinated Sic1 generated *in vitro* with recombinant SCF^{Cdc4} is degraded by affinity-purified yeast proteasomes (Verma *et al*., 2001). Remarkably, purified proteasomes can extract ubiquitinated Sic1 from complexes with S phase cyclin-Cdk, degrade the Sic1 and release active S phase cyclin-Cdk protein kinase. This result emphasized that the proteasome has the intrinsic ability to disassemble protein complexes to selectively degrade ubiquitinated substrates, and set the stage for our subsequent studies on substrate targeting and deubiquitination.

Role of deubiquitination in the degradation of Sic1: In the course of characterizing the degradation of ubiquitinated Sic1, we noticed that when the 20S protease inhibitor epoxomicin was present, ubiquitinated Sic1 was converted to a completely deubiquitinated species (Verma *et al*., 2002). Fortuitously, at about the same time as this we observed that the CSN – which is related to the lid subcomplex of the proteasome 19S cap – cleaves the ubiquitin-like protein Nedd8 from the Cul1 subunit of SCF. Spurred by this confluence of observations, we demonstrated that the Rpn11 subunit of the proteasome lid contains a putative JAMM metalloprotease active site analogous to that of Csn5, and this motif is essential for the deubiquitination of Sic1 *in vitro* and the degradation of multiple UPS substrates *in vivo*.

Multiubiquitin chain receptors target substrate to the proteasome: Although it has long been clear that a multiubiquitin chain targets an appended substrate to the proteasome for degradation, the mechanism of targeting has remained controversial. Genetic studies in yeast have suggested a potential role for multiubiquitin chain-binding proteins, including Rad23, Dsk2, Ddi1, and Rpn10. In contrast, biochemical studies in mammalian systems have emphasized a role for the proteasome ATPase Rpt5 as a multiubiquitin chain receptor, and have suggested that proteins such as Rad23 prevent premature metabolism of substrate-linked ubiquitin chains. We reasoned that our reconstituted system would enable us to address this fundamental question from a functional, mechanistic perspective. We first demonstrated using mutant proteasomes and add-back experiments that Rad23 and Rpn10 play a direct role in targeting ubiquitinated Sic1 to the proteasome for degradation (Verma *et al*., 2004a). We then went on to show that the multiubiquitin chain receptor activities of Rad23 and Rpn10 play a redundant role in sustaining turnover of Sic1 *in vivo*. Surprisingly, individual deletion of these and other receptor proteins led to the accumulation of different UPS substrates, suggesting that the receptors define a layer of specificity that resides downstream of the E3s and upstream of the proteasome. This hypothesis opens up a host of interesting questions

about how specificity is achieved in the targeting step, and what its biological purpose is. We plan to address these key questions over the next several years using a combination of biochemical, molecular genetic, and proteomic approaches.

In a parallel effort, we demonstrated that the substrate-targeting step can be blocked by a small molecule that emerged from a chemical genetic screen conducted in the lab of our collaborator, Dr. Randy King. We demonstrated that this compound, which we named 'ubistatin A,' binds to the ubiquitin chain in the same intersubunit cleft that is normally bound by the multiubiquitin chain receptors (Verma *et al*., 2004b). We believe that ubistatins will be useful tools for studies on the UPS.

Role of Cdc48 in targeting and degradation of ubiquitinated proteins: Recently, we have become intrigued by a poorly understood protein, Cdc48, that, like the ubiquitin chain receptors, operates downstream of ubiquitin ligases to promote degradation of ubiquitinated proteins by the proteasome. The role of Cdc48 in protein turnover was originally thought to be confined to pulling malfolded secretory proteins through the endoplasmic reticulum membrane so that they can be degraded by the proteasome. However, several lines of evidence hint at a far broader role. Interestingly, there may be as many as seven distinct Cdc48 complexes in budding yeast, and unpublished data from my lab points to the existence of ~15 distinct Cdc48 complexes in human cells (see abstract by G. Alexandru). Why all of this complexity? It is difficult to even begin to answer this question, because we know so little about Cdc48's function apart from its role in translocation across the ER membrane. Whatever Cdc48 is doing, it appears to be a fundamental component of the UPS, and thus, understanding how it works is important. We plan to attack this problem by first identifying substrates whose degradation depends on particular Cdc48 complexes by employing the mass spec-based proteomics technology discussed below (see abstract by J. Graumann). We will then reconstitute the degradation of these substrates using defined components. The objective will be to develop a reconstituted system in which turnover of the substrate is dependent upon Cdc48. We will then use this system to establish the mechanism-of-action of Cdc48. Armed with this information, we will be in a position to initiate investigations on how ubiquitin receptors and Cdc48 complexes collaborate to enable degradation of ubiquitinated substrates. Given the diversity of receptors that guide ubiquitinated proteins to the proteasome and the diversity of Cdc48 complexes that appear to act in concert with these receptors, there is clearly much about the targeting and degradation of ubiquitinated proteins that we do not understand, and thus, this topic may be fertile ground for making unexpected discoveries.

Proteomics

The Yates laboratory at Scripps has developed a method dubbed, "multidimensional protein identification technology" (MudPIT). In this method, a complex mixture of proteins is digested with protease and the resulting peptides are fractionated by two-dimensional chromatography on a column that is in-line with the electrospray interface of a mass spectrometer. Johannes Graumann and Thibault Mayor have been applying MudPIT to the study of ubiquitination in yeast. As an example of this approach, we have used subtractive comparisons of samples from wild-type and mutant cells to identify the ubiquitinated polypeptides that accumulate when the Rpn10 multiubiquitin chain receptor is absent (see abstract by T. Mayor). During the next few years, we plan to more fully integrate multidimensional mass spectrometry into ongoing projects in the lab. I believe that this technology will allow us to ask questions that we have never been able to ask before, and will also provide us with a new perspective. Our initial focus will be to marry our approach with quantification methods to systematically identify proteins that are targets of the various multiubiquitin chain receptors encoded in the yeast genome. By identifying the set of substrates whose abundance is altered when a particular ubiquitin pathway component is mutated or blocked by the action of a drug, we can gain insight into enzyme-substrate relationships, which in turn may yield insights into the mechanisms that underlie specificity. Moreover, knowledge of the substrates affected can provide clues to the phenotypes that may occur upon inactivation of a particular component. Finally, the ability to quantify substrate accumulation may enable us to see subtle defects, such as those that occur when one member of a redundant pair of enzymes is mutated.

Functions of the RENT complex in cell cycle control and nucleolar biogenesis

Several years ago, a graduate student, Wenying Shou, discovered the RENT complex, and proposed that the mitotic exit network (MEN) specifies the exit from mitosis in budding yeast by promoting disassembly of RENT (Shou *et al*., 1999). RENT is comprised of the nucleolar anchor protein Net1, the cell cycle regulatory protein phosphatase Cdc14 and the chromatin silencing protein Sir2. Cdc14 is required for the exit from mitosis, which it promotes by dephosphorylating (and thereby activating) proteins that mediate the inactivation of cyclin/CDK activity at the end of mitosis. Throughout the cell cycle, Cdc14 is confined to the nucleolus through its interaction with Net1. At the end of mitosis, the successful completion of anaphase activates the MEN signaling pathway, which disengages Cdc14 from Net1. The emancipated Cdc14 goes on to inactivate cyclin/CDK and thereby trigger the exit from mitosis. This hypothesis for how the exit from mitosis is controlled in budding yeast was dubbed 'RENT control' by Shou, *et al*., 1999. Over the past few years, it has become apparent that RENT is disassembled by a two-step mechanism. In early anaphase, Cdc14 is released from Net1 through a novel activity of separase. Separase is a protease that activates chromosome segregation in anaphase by cleaving the cohesin protein that holds sister chromatids together.

Throughout interphase and early mitosis, separase activity is repressed by a tightly bound inhibitor, securin. At the metaphase-anaphase boundary, securin is abruptly degraded, thereby liberating separase to cleave cohesin and initiate chromosome segregation. In addition to being a protease, separase has a second activity that promotes the phosphorylation of Net1 by cyclin B–Cdk. A graduate student in the lab, Ramzi Azzam, had identified this phosphorylation and demonstrated that it induces the dissociation of Cdc14 from Net1 (Azzam *et al*., 2004). Thus, the action of separase links initiation of the exit from mitosis with the initiation of chromosome segregation. In late anaphase the MEN serves to sustain Cdc14 release such that its substrates are dephosphorylated and the cell exits mitosis. Our current goal is to understand how the terminal signaling component of the MEN, the protein kinase Dbf2, mediates the sustained release of Cdc14 from the nucleolus, thereby triggering exit from mitosis.

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227. p97 regulation via interaction with a variety of p47-related co-factors

Gabriela M. Alexandru

The 97 kDa valosin-containing protein (p97 or VCP) is a type II AAA (ATPase associated with a variety of activities) ATPase. AAA-ATPases are highly conserved from archaebacteria to mammals. p97 plays a role in seemingly unrelated cellular activities, such as membrane fusion, endoplasmic reticulum-associated degradation (ERAD) and cell cycle regulation. All of these functions involve recognition of ubiquitinated protein substrates and, at least in some cases, their subsequent degradation by the proteasome. In its active form, p97 forms homohexameric barrel structures in which the N-termini are free to bind specific co-factors. Thus, p97 in complex with p47 is thought to regulate membrane fusion, while p97/Npl4/Ufd1 complexes are mainly required for ERAD. In an attempt to further understand the molecular basis of p97's diverse functions we have analyzed p97 immunoprecipitates from human tissue culture cells by MuDPIT mass spectrometry, searching for new p97 co-factors. This analysis revealed a whole group of previously unidentified p97-binding partners, all sharing a domain structure similar at least in part to p47. Two of them have been linked to human diseases, such as atopic dermatitis and alveolar soft part sarcoma. However, the biological function for most of these proteins is largely unknown. Comparative analysis of immunoprecipitates from human cells transfected with a Flag-tagged version of each of the newly identified co-factors revealed that some of them can bind ubiquitinated proteins and also interact with the ERAD p97 co-factors, Npl4 and Ufd1. Their possible involvement in ERAD will be investigated by

checking the effect of their siRNA depletion on the degradation of various ERAD model substrates.

228. Regulation of SCF ubiquitin ligase activity

Ethan Emberley

Ubiquitin-mediated protein degradation has emerged as a pivotal process in many areas of cell biology. Members of the superfamily of multi-subunit CRL (cullin-RING ligase) ubiquitin ligase enzymes transfer ubiquitin molecules onto target proteins destined for degradation by the 26S proteosome. Despite impressive advancements in our knowledge about CRLs, including the prototypical CRL, SCF, many fundamental questions remain about the mechanism of action and regulation of these key enzymes. We have begun to study whether substrate binding to its respective substrate receptor subunit might control CRL activity. We propose that substrate binding either positively regulates the attachment of the ubiquitin-like protein Nedd8 to the cullin subunit of CRLs by Ubc12, or negatively regulates cullin deneddylation by the COP9 signalosome. Neddylation of cullin is necessary for activity of SCF and other CRLs, and provides an important mechanism by which these enzymes may be regulated. Our results suggest that substrate binding promotes neddylation of the associated cullin molecule, which may serve as a feedback mechanism that links the activity of a CRL to the level of available substrate for that particular enzyme.

229. Role of ubiquitination in targeting protein substrates to the 26S proteasome

Nazli Ghaboosi

The ubiquitin-dependent degradation pathway begins with the activation of ubiquitin by the E1 ubiquitin-activating enzyme. The ubiquitin moiety is transferred to one of several E2 ubiquitin-conjugating enzymes and is subsequently attached to the substrate with the aid of an E3 ubiquitin ligase. The multi-ubiquitinated substrate is then targeted for destruction by the 26S proteasome through a poorly understood mechanism. While the list of known E2s, E3s, and substrates is steadily growing, there is only one E1 gene in all somatic eukaryotic cell types. At the apex of this intricate network, E1 offers a unique perspective from which to address the many unanswered questions regarding the physiological roles of ubiquitin conjugation.

We created a temperature-sensitive mutant of the essential yeast E1 gene, *UBA1*, using random PCR mutagenesis. The resulting mutant strain, *uba1 204*, allows us to conditionally disrupt the entire downstream ubiquitination pathway. At the restrictive temperature, mutant cells exhibit rapid loss of all detectable ubiquitin-protein conjugates, as well as stabilization of diverse ubiquitin pathway substrates. These cells also have the hallmarks of other ubiquitin pathway mutants, such as cell cycle arrest and sensitivity to cellular stress.

Using this novel yeast mutant, we are examining the effect that ubiquitin activation has on substrate recognition and interaction of substrate receptors with the proteasome. Recent work in the lab showed that multiubiquitin chain-binding proteins (MCBPs) such as Rad23, Dsk2, and Rpn10 function as receptors to selectively recruit substrates to the proteasome (Verma *et al.*, 2004). While the ubiquitin activation and conjugation defects in *uba1 204* do not affect proteasome assembly or activity, we have shown that they do influence how the proteasome interacts with some MCBPs. Our findings indicate the presence of ubiquitinated substrates activates targeting of MCBPs to the proteasome.

Reference

Verma *et al.* (2004) *Cell* **118**(1):99-110.

230. Using MudPIT to characterize components of the proteasome pathway in yeast

Johannes Graumann

Multidimensional protein identification technology (MudPIT) is a method developed by the laboratory of John Yates at Scripps to analyze complex protein mixtures. In MudPIT, a sample is digested with protease and the resulting peptides are separated on a multidimensional capillary column that is in-line with the electorspray interface of an ion trap mass spectrometer[1]. As a first test of the utility of MudPIT for discovery applications in cell biology, we applied this technology to the proteomic characterization of multiple components of a cellular reaction pathway - i.e., pathway proteomics[2].

Recent work in the lab[3] has revealed a novel layer of substrate specificity in the ubiquitin-proteasome system. Ubiquitinated proteins bind specific receptor proteins, which help guide the substrate to the proteasome for proteolysis. By performing MudPIT analysis of immunoprecipitated proteasomes from wild-type cells and mutants lacking specific substrate receptor proteins, we hope to shed light on the mechanism by which different substrates are sorted to different receptors.

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231. Molecular mechanism of multiubiquitin chain synthesis by Cdc34

Gary Kleiger

Cdc34 is a ubiquitin-conjugating enzyme that is an essential component of the Ubiquitin Proteasome System (UPS). It functions by catalyzing the formation of polyubiquitin chains on protein substrates destined for degradation by the 26S proteasome. Cdc34 works in concert with the ubiquitin ligase SCF, which is also an essential component of the UPS.

Cdc34 is a processive enzyme that shows at least 10-fold greater activity for producing K48-linked poly-ubiquitin chains than all other ubiquitin chain linkages combined. One potential explanation for this processivity is that Cdc34 contains a ubiquitin-binding site on its surface that places K48 near the active site. To test this

hypothesis, we have used molecular docking simulations to generate a model of the Cdc34-ubiquitin complex. From this model, residues at the intermolecular interface were chosen such that mutations would disrupt the interaction. Several biophysical assays are being employed to both measure the binding affinity and confirm the orientations of Cdc34 and ubiquitin in the complex, including surface plasmon resonance, NMR and chemical cross-linking. If the model is correct, mutation of residues at the intermolecular interface will reduce or eliminate ubiquitin binding. Mutations that have an effect on ubiquitin binding will be tested for Cdc34 function using specific Cdc34 activity assays.

Detailed molecular analysis of the Ubc7 (Cdc34 and Ubc7 are closely related both in function and in structure) active site for residues involved in the catalysis of isopeptide bond formation identified a conserved histidine found in all Cdc34 and Cdc34-like amino acid sequences. We hypothesized that this residue is involved in either stabilization of the transition state complex or deprotonation of the incoming epsilon amino group on the lysyl side chain that attacks the donor ubiquitin thioesterified to Cdc34. Mutation of this residue to alanine resulted in a defect in a Sic1 ubiquitination assay. This mutant will be further characterized to determine its role in Cdc34 catalysis.

232. The roles of ubiquitin-mediated proteolysis in transcription

Rusty Lipford

The ubiquitin-proteasome system (UPS) plays numerous diverse roles in the regulation of transcription. We are studying the impact of the UPS on the function of yeast transcriptional activators, like Gcn4 and Gal4, which are targeted for ubiquitination and degradation. Previous studies of Gcn4 established that this activator of amino acid biosynthesis genes is a target for SCF-mediated ubiquitination and proteolysis. In addition, these studies illuminated a role in Gcn4 ubiquitination for the cyclin-dependent kinase, Srb10, a component of the mediator complex of the RNA polymerase II holoenzyme. These findings suggest that the transcription machinery and, therefore, the transcription process are coupled to turnover of the activator. To characterize further this coupling we have determined that Srb10-dependent phosphorylation and subsequent ubiquitination and proteolysis of Gcn4 require promoter association and transcriptional activation function. In addition multiple transcription initiation mutants disrupt phosphorylation and turnover of Gcn4.

On the other side of the coupling between transcription and turnover, we have now demonstrated that inhibition of the UPS at any step (e.g., ubiquitin expression, E2 function, E3 function, or proteasome function) reduces the transcriptional activity of Gcn4 and Gal4. In fact, compromised UPS function appears to prevent the association of RNA polymerase II with target genes despite the accumulation of excess activator at their promoters. In addition, the transcriptional activity of stable versions of Gcn4 that lack CDK phosphorylation is largely insensitive to UPS impairment. These findings suggest that CDK-phosphorylation of Gcn4 creates a requirement for subsequent turnover to promote normal levels of transcriptional activation. We call this mode of activator regulation, where turnover is required to fully stimulate a reaction, "activation by destruction." Such a mode appears to be a common regulatory motif involved in numerous normal and disease-related cellular processes.

233. Profiling of ubiquitylated proteins by quantitative mass spectrometry to study the UPS

Thibault Mayor

Ubiquitylation is one of the most common posttranslational modifications in eukaryotic cells and a major goal in proteomics is to catalog these modifications and understand their role. A classical function of ubiquitylation is to target substrates for degradation via the ubiquitin-proteasome system (UPS). Here, we employ a method in which affinity-purified ubiquitin conjugates arising from experimentally perturbed and reference cultures of *Saccharomyces cerevisiae* were differentiated by labeling with ^{14}N and ^{15}N isotopes. Multidimensional mass spectrometry (LC/LC-MS/MS) coupled with ratiometric quantification of 'heavy' and 'light' peptides revealed ubiquitylated proteins that accumulated or became de-enriched in response to a particular treatment. By adding to one of the two cell populations a proteasomal inhibitory drug, we identified over 280 proteins that specifically accumulated in treated cells, hence are UPS substrates. We extended our analysis to mutants of the Rpn10 UPS-delivery pathway. Rpn10 is a proteasome receptor protein that specifically promotes the degradation of some ubiquitylated substrates. To what extent Rpn10 is involved in targeting of UPS substrates was not known. We, therefore, compared wild-type vs. *rpn10*Δ cells - using ^{14}N and ^{15}N labeling– and observed an elevated accumulation of ubiquitylated forms of ~40% of the proteasomal substrates. We then further analyzed Rpn10 function by selectively deleting its ubiquitin-binding UIM domain and only as many as one-third of proteins accumulated. To our knowledge, these represent the first physiological UPS substrates that have been shown to be affected by loss of the UIM domain. This also confirms that the dominant function of Rpn10 is provided by its VWA domain. This approach illustrates the feasibility of systems-level quantitative analysis of enzyme-substrate networks in the UPS that can be now used for identifying targets of specific ubiquitin ligases and DUB enzymes.

234. Regulation of *Saccharomyces cerevisiae* Cdc14

Dane Mohl

Our work hopes to illuminate the mechanisms that monitor the duplication of the genome, and link completion of chromosome segregation to the initiation of cell division. In my research I have used the model yeast system *S. cerevisiae* to look more closely at the regulation of a key cell cycle phosphatase, Cdc14. Our work has demonstrated that a protein complex called Dbf2/Mob1,

that becomes active when each of the two new nuclei are segregated to opposite compartments of a pre-divisional cell, regulates a Cdc14 phosphatase complexed with Net1p thereby linking genome duplication to cytokinesis.

The aim of my project has been the identification of *cis*-acting sequences within Cdc14 and its inhibitor Net1p that are required for properly regulating the stability of this complex in late mitosis. We have also been searching for the transacting factors that act upon key regulatory sequences in both proteins. Live cell imaging and GFP fluorescence localization led us to conclude that a small portion of Cdc14-GFP visited the daughter bound spindle pole bodies of anaphase cells, and could therefore, be a direct substrate of Dbf2/Mob1.

235. Targeting cancer-promoting proteins for ubiquitination and degradation

Agustin Rodriguez

We are developing a new approach to cancer therapy that exploits the ubiquitin-dependent proteolytic system of eukaryotic cells. This approach employs cell-permeable molecules that bind to the substrate-docking site of an ubiquitin ligase. Covalently linking such a molecule to compounds that bind to a desired target protein yields a heterobifunctional compound that we refer to as a ProTac (Proteolysis Targeting Chimeric Pharmaceuticals). Protacs can be used, in theory, to trigger the destruction of any protein for which there exists a small, cell-permeable ligand. As "proof of principle," we designed a ProTac that contains a peptide that binds with high affinity to the substrate-docking domain of the ubiquitin ligase $SCF^{\beta\text{-}TRCP}$. We then chemically linked this peptide to the small molecule ovalicin, which binds covalently and specifically to the cellular enzyme methionine aminopeptidase-2 (MetAP-2). We demonstrated that the resulting peptide-ovalicin (ProTac) tethers MetAP-2 to $SCF^{\beta\text{-}TRCP}$, and targets MetAP-2 for ubiquitination and degradation[1]. To determine whether ProTacs could recruit different substrates to the $SCF^{\beta\text{-}TRCP}$ ubiquitin ligase for ubiquitination through non-covalent interactions, we generated ProTacs containing the same ligase-binding peptide linked to either estradiol or testosterone. Both the estrogen receptor (ER) and androgen receptor (AR) have been shown to enhance growth of breast and prostate cancer, respectively. We demonstrated that Protacs containing estradiol or dihydroxytestosterone can trigger the degradation of ER and AR, respectively[2]. Making a cell-permeable ProTac was the next step to induce degradation of a target in living cells. The latest-generation ProTaCs are based on a hydroxyproline-containing peptide from HIF-1 that binds to the VHL ubiquitin ligase complex. These new ProTaCs based on the HIF-1 peptide induced the degradation of AR and ER in tissue culture cells[3]. Induced degradation of ER arrested cell proliferation of estrogen-dependent breast cancer cells as a consequence of down regulation of ER signaling. On the other hand, breast cancer cell lines that do not express ER and are not dependent upon ER signaling for proliferation were not affected by treatment with ProTaCs. We are now focusing on developing ProTacs into effective drugs for cancer treatment *in vivo*.

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236. Mechanism of ubiquitin chain assembly by SCF ligases

Anjanabha Saha

Protein modification by ubiquitination is mediated by ubiquitin ligases (E3) that include the highly diverse and extensive superfamily of cullin-RING ligases (CRLs). CRLs plays a central role in various cellular processes. We are utilizing the human SCF complex to understand how the dynamics of substrate and E2 (ubiquitin conjugating enzyme) association with E3 regulate the specificity and the processivity of ubiquitination. These studies will aid in establishing how SCF distinguishes substrates from non-substrates and provide insight into how SCF ligases achieve specificity *in vivo*. In addition, we are also examining how covalent modification of CRLs by Nedd8 affects the kinetics of substrate ubiquitination.

237. Determining the requirements for proteolysis by purified 26S proteasomes

Rati Verma, Robert Oania

Labile substrates of the 26S proteasome are earmarked for proteolysis by the covalent attachment of a multiubiquitin chain on acceptor lysines. Ubiquitinated proteins are then recruited to the 26S proteasome, where they are destroyed. We have recently shown that the multiubiquitin chain-binding proteins (MCBPs) Rad23 and Rpn10 function as receptors to recruit substrates to the proteasome[*]. Under physiological conditions, the receptors display selectivity in substrate targeting, even though all substrates presumably contain the same universal targeting signal-a tetraubiquitin chain. We are currently investigating the molecular basis of this selectivity. In addition, we are also investigating specific putative receptor pathways, namely those involving Cdc48.

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Summary: In eukaryotic cells, the cyclin-dependent kinases (Cdks) control the progression of the cell cycle by regulating the accurate replication of the genome during S-phase and the faithful segregation of the chromosomes at mitosis (M-phase). The entry into these phases of the cell cycle is controlled by Cdks called S-phase promoting factor (SPF) and M-phase promoting factor (MPF). The action of these Cdks must be controlled both temporally and spatially in a very stringent manner. This strict regulation is imparted by a number of checkpoint mechanisms. For example, cells containing unreplicated DNA cannot enter mitosis due to the mobilization of the replication checkpoint. The Dunphy laboratory is engaged in the elucidation of the molecular mechanisms underlying the regulation of SPF and MPF during the cell cycle. Most of these experiments are conducted with *Xenopus* egg extracts, a system in which the entire cell cycle can be reconstituted *in vitro*.

The first member of the cyclin-dependent protein kinase family described is M-phase promoting factor (MPF), which contains the Cdc2 protein kinase and a regulatory subunit known as cyclin B. Since the identification of the molecular components of MPF, there has been rapid and extensive progress in unraveling the biochemistry of mitotic initiation. It is now well established that MPF acts by phosphorylating a myriad of structural and regulatory proteins that are involved directly in mitotic processes such as nuclear membrane disintegration, chromosome condensation, and mitotic spindle assembly. An ongoing challenge to the cell cycle field is the elucidation of how these phosphorylation reactions regulate the structural and functional properties of the various targets of MPF.

We have been most interested in how the cyclin-dependent protein kinases are regulated during the cell cycle. The principal focus of our laboratory has been on the regulatory mechanisms that govern the activation of MPF at the G2/M transition. Some immediate and long-term issues that we are tackling include: (1) What controls the timing of MPF activation so that it occurs at a defined interval following the completion of DNA replication; (2) How do various checkpoint or feedback controls influence the Cdc2/cyclin B complex; and, (3) What are the molecular differences between the simple biphasic cell cycle found in early embryonic cells and the more complex cell cycles that arise later in development?

More recently, we have been able to study at the molecular level some of the key events leading to the initiation of DNA replication at the G1/S transition. These events involve a cooperative interaction between the Origin Recognition Complex (ORC), the Cdc6 protein, and members of the Mcm family. These studies may ultimately help us understand how S-phase and M-phase are integrated with one another.

In principle, the regulation of cyclin-dependent kinases such as MPF could occur at any of several levels, including synthesis of the cyclin protein, association between the Cdc2 and cyclin proteins, or posttranslational modification of the Cdc2/cyclin complex. The posttranslational regulation of the Cdc2/cyclin complex is particularly important, even in early embryonic cells that manifest the simplest cell cycle programs. In recent years, many of the elaborate details of this Cdc2 modification process have been defined. For example, the binding of cyclin results in three phosphorylations of Cdc2: one at threonine 161 that is required for Cdc2 activity, and two dominantly inhibitory phosphorylations at threonine 14 and tyrosine 15. A variety of genetic and biochemical experiments have established that the inhibitory tyrosine phosphorylation of Cdc2 is an especially important mechanism of cell cycle regulation. As described in greater detail below, there is now strong evidence that the decision to enter mitosis involves considerably more than the tyrosine dephosphorylation of Cdc2. However, a thorough understanding of the kinase/phosphatase network that controls the phosphotyrosine content of Cdc2 will provide a firm foundation for understanding other facets of mitotic regulation.

Our laboratory has made substantial contributions to understanding the molecular mechanisms controlling the activation of the Cdc2 protein. For our studies, we utilize cell-free extracts from *Xenopus* eggs. Due to pioneering work in a number of the laboratories, it is now possible to re-create essentially all of the events of the cell cycle in these extracts. Consequently, it is feasible to study the molecular mechanisms of Cdc2 regulation in intricate detail with this experimental system. To facilitate these studies, we make extensive use of recombinant DNA technology to overproduce cell cycle proteins in either bacteria or baculovirus-infected insect cells. Moreover, in conjunction with our biochemical studies, we are taking advantage of the fission yeast system to exploit genetic approaches to identify novel *Xenopus* regulators of the cell cycle.

238. Claspin, a Chk1-regulatory protein, monitors DNA replication on chromatin independently of RPA, ATR, and Rad17

Joon Lee, Akiko Kumagai, William G. Dunphy

Claspin is required for the ATR-dependent activation of Chk1 in *Xenopus* egg extracts containing incompletely replicated DNA. We show here that Claspin associates with chromatin in a regulated manner during S-phase. Binding of Claspin to chromatin depends on the pre-replication complex (pre-RC) and Cdc45 but not on

replication protein A (RPA). These dependencies suggest that binding of Claspin occurs around the time of initial DNA unwinding at replication origins. By contrast, both ATR and Rad17 require RPA for association with DNA. Claspin, ATR, and Rad17 all bind to chromatin independently. These findings suggest that Claspin plays a role in monitoring DNA replication during S-phase. Claspin, ATR, and Rad17 may collaborate in checkpoint regulation by detecting different aspects of a DNA replication fork.

239. Phosphorylated Claspin interacts with a phosphate-binding site in the kinase domain of Chk1 during ATR-mediated activation

Seong Yun Jeong[*], Akiko Kumagai, Joon Lee, William G. Dunphy

Claspin is essential for the ATR-dependent activation of Chk1 in *Xenopus* egg extracts containing incompletely replicated or UV-damaged DNA. The activated form of Claspin contains two repeated phosphopeptide motifs that mediate its binding to Chk1. We show that these phosphopeptide motifs bind to Chk1 by means of its N-terminal kinase domain. The binding site on Chk1 involves a positively charged cluster of amino acids that contains lysine 54, arginine 129, threonine 153, and arginine 162. Mutagenesis of these residues strongly compromises the ability of Chk1 to interact with Claspin. These amino acids lie within regions of Chk1 that are involved in various aspects of its catalytic function. The predicted position on Chk1 of the phosphate group from Claspin corresponds to the location of activation-loop phosphorylation in various kinases. In addition, we have obtained evidence that the C-terminal regulatory domain of Chk1, which does not form a stable complex with Chk1 under our assay conditions, nonetheless has some role in Claspin-dependent activation. Overall, these results indicate that Claspin docks with a phosphate-binding site in the catalytic domain of Chk1 during activation by ATR. Phosphorylated Claspin may mimic an activating phosphorylation of Chk1 during this process.

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240. Adaptation of a DNA replication checkpoint response depends upon inactivation of Claspin by the Polo-like kinase

Hae Yong Yoo, Akiko Kumagai, Anna Shevchenko, Andrej Shevchenko, William G. Dunphy

The checkpoint mediator protein Claspin is essential for the ATR-dependent activation of Chk1 in *Xenopus* egg extracts containing aphidicolin-induced DNA replication blocks. We show that during this checkpoint response Claspin becomes phosphorylated on threonine-906 (T906), which creates a docking site for Plx1, the *Xenopus* Polo-like kinase. This interaction promotes the phosphorylation of Claspin on a nearby serine (S934) by Plx1. After a prolonged interphase arrest, aphidicolin-treated egg extracts typically undergo adaptation and enter into mitosis despite the presence of incompletely replicated DNA. In this process, Claspin dissociates from chromatin and Chk1 undergoes inactivation. By contrast, aphidicolin-treated extracts containing mutants of Claspin with alanine substitutions at positions 906 or 934 (T906A or S934A) are unable to undergo adaptation. Under such adaptation-defective conditions, Claspin accumulates on chromatin at high levels and Chk1 does not decrease in activity. These results indicate that the Plx1-dependent inactivation of Claspin results in termination of a DNA replication checkpoint response.

241. Mcm2 is a direct substrate of ATM and ATR during DNA damage and DNA replication checkpoint responses

Hae Yong Yoo, Anna Shevchenko, Andrej Shevchenko, William G. Dunphy

In vertebrates, ATM and ATR are critical regulators of checkpoint responses to damaged and incompletely replicated DNA. These checkpoint responses involve the activation of signaling pathways that inhibit the replication of chromosomes with DNA lesions. In this report, we describe isolation of a cDNA encoding a full-length version of *Xenopus* ATM. Using antibodies against the regulatory domain of ATM, we have identified the essential replication protein Mcm2 as an ATM-binding protein in *Xenopus* egg extracts. *Xenopus* Mcm2 undergoes phosphorylation on serine 92 (S92) in response to the presence of double-stranded DNA breaks or DNA replication blocks in egg extracts. This phosphorylation involves both ATM and ATR, but the relative contribution of each kinase depends upon the checkpoint-inducing DNA signal. Furthermore, both ATM and ATR phosphorylate Mcm2 directly on S92 in cell-free kinase assays. Immunodepletion of both ATM and ATR from egg extracts abrogates the checkpoint response that blocks chromosomal DNA replication in egg extracts containing double-stranded DNA breaks. These experiments indicate that ATM and ATR phosphorylate the functionally critical replication protein Mcm2 during checkpoint responses that prevent the replication of abnormal chromosomes.

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242. Claspin and the activated form of ATR-ATRIP collaborate in the activation of Chk1

Akiko Kumagai, Soo Mi Kim, William G. Dunphy

Claspin is necessary for the ATR-dependent activation of Chk1 in *Xenopus* egg extracts containing incompletely replicated DNA. ATR possesses a regulatory partner called ATRIP. We have studied the respective roles of ATR-ATRIP and Claspin in the activation of Chk1. ATR-ATRIP binds well to various DNA templates in *Xenopus* egg extracts. ATR-ATRIP bound to a single-stranded DNA template is weakly active. By contrast, the ATR-ATRIP complex on a DNA template containing both single-stranded and double-stranded regions displays a large increase in kinase activity. This observation suggests that ATR-ATRIP normally undergoes activation upon association with specific nucleic acid structures at DNA replication forks. Without Claspin, activated ATR-ATRIP phosphorylates Chk1 weakly in a cell-free reaction. Addition of Claspin to this reaction strongly stimulates the phosphorylation of Chk1 by ATR-ATRIP. Claspin also induces significant autophosphorylation of Chk1 in the

absence of ATR-ATRIP. Taken together, these results indicate that the checkpoint-dependent phosphorylation of Chk1 is a multi-step process involving activation of the ATR-ATRIP complex at replication forks and presentation of Chk1 to this complex by Claspin.

243. Roles of replication fork-interacting and Chk1-activating domains from Claspin in a DNA replication checkpoint response

Joon Lee, Daniel A. Gold, Anna Shevchenko, Andrej Shevchenko, William G. Dunphy

Claspin is essential for the ATR-dependent activation of Chk1 in *Xenopus* egg extracts containing incompletely replicated DNA. Claspin associates with replication forks upon origin unwinding. We show that Claspin contains a replication fork-interacting domain (RFID, residues 265-605) that associates with Cdc45, DNA polymerase epsilon, RPA, and two RFC complexes on chromatin. The RFID contains two basic patches (BP1 and BP2) at amino acids 265-331 and 470-600, respectively. Deletion of either BP1 or BP2 compromises optimal binding of Claspin to chromatin. Absence of BP1 has no effect on the ability of Claspin to mediate activation of Chk1. By contrast, removal of BP2 causes a large reduction in the Chk1-activating potency of Claspin. We also find that Claspin contains a small Chk1-activating domain (CKAD, residues 776-905) that does not bind stably to chromatin, but is fully effective at high concentrations for mediating activation of Chk1. These results indicate that stable retention of Claspin on chromatin is not necessary for activation of Chk1. Instead, our findings suggest that only transient interaction of Claspin with replication forks potentiates its Chk1-activating function. Another implication of this work is that stable binding of Claspin to chromatin may play a role besides the activation of Chk1.

244. Phosphorylation of Chk1 by ATR in *Xenopus* egg extracts requires binding of ATRIP to ATR but not the stable DNA-binding or coiled-coil domains of ATRIP

Soo Mi Kim, Akiko Kumagai, Joon Lee, William G. Dunphy

ATR, a critical regulator of DNA replication and damage checkpoint responses, possesses a binding partner called ATRIP. We have studied the functional properties of *Xenopus* ATR and ATRIP in incubations with purified components and in frog egg extracts. In purified systems, ATRIP associates with DNA in both RPA-dependent and RPA-independent manners, depending on the composition of the template. However, in egg extracts, only the RPA-dependent mode of binding to DNA can be detected. ATRIP adopts an oligomeric state in egg extracts that depends upon binding to ATR. In addition, ATR and ATRIP are mutually dependent on one another for stable binding to DNA in egg extracts. The ATR-dependent oligomerization of ATRIP does not require an intact coiled-coil domain in ATRIP and does not change in the presence of checkpoint-inducing DNA templates. Egg extracts containing a mutant of ATRIP that cannot bind to ATR are defective in the phosphorylation of Chk1. However, extracts containing mutants of ATRIP lacking stable DNA-binding and coiled-coil domains show no reduction in the phosphorylation of Chk1 in response to defined DNA templates. Furthermore, activation of Chk1 does not depend upon RPA under these conditions. These results suggest that ATRIP must associate with ATR in order for ATR to carry out the phosphorylation of Chk1 effectively. However, this function of ATRIP does not involve its ability to mediate the stable binding of ATR to defined checkpoint-inducing DNA templates in egg extracts, does not require an intact coiled-coil domain, and does not depend on RPA.

245. TopBP1 activates the ATR-ATRIP complex

Akiko Kumagai, Joon Lee, Hae Yong Yoo, William G. Dunphy

ATR is a key regulator of checkpoint responses to incompletely replicated and damaged DNA, but the mechanisms underlying control of its kinase activity are unknown. TopBP1, the vertebrate homolog of yeast Cut5/Dpb11, has dual roles in initiation of DNA replication and regulation of checkpoint responses. We show that recombinant TopBP1 induces a large increase in the kinase activity of both *Xenopus* and human ATR. The ATR-activating domain resides in a conserved segment of TopBP1 that is distinct from its numerous BRCT repeats. The isolated ATR-activating domain from TopBP1 induces ectopic activation of ATR-dependent signaling in both *Xenopus* egg extracts and human cells. Furthermore, *Xenopus* egg extracts containing a version of TopBP1 with an inactivating point mutation in the ATR-activating domain are defective in checkpoint regulation. These studies establish that activation of ATR by TopBP1 is a crucial step in the initiation of ATR-dependent signaling processes.

246. Site-specific phosphorylation of a checkpoint mediator protein controls its responses to different DNA structures

Hae Yong Yoo, Seong Yun Jeong, William G. Dunphy*

The checkpoint mediator protein Claspin is indispensable for the ATR-dependent phosphorylation of Chk1 in response to stalled DNA replication forks in *Xenopus* egg extracts. We show that Claspin also participates in the detection of chromosomal double-stranded DNA breaks (DSBs) in this system. Significantly, removal of Claspin from egg extracts only partially abrogates the activation of Chk1 in response to chromatin with DSBs, whereas depletion of both Claspin and BRCA1 completely abolishes this activation. The function of Claspin in this DSB-triggered pathway depends upon phosphorylation of T817 and S819 by ATR. Conversely, neither phosphorylation of Claspin on these sites nor the presence of BRCA1 is necessary for activation of Chk1 in response to stalled replication forks. Thus, site-specific phosphorylation of a checkpoint mediator protein is a crucial determinant in the discrimination between various checkpoint-inducing structures. Furthermore, checkpoint mediator proteins exhibit functional overlap that varies depending on the nature of the checkpoint-triggering DNA signal.

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Publications

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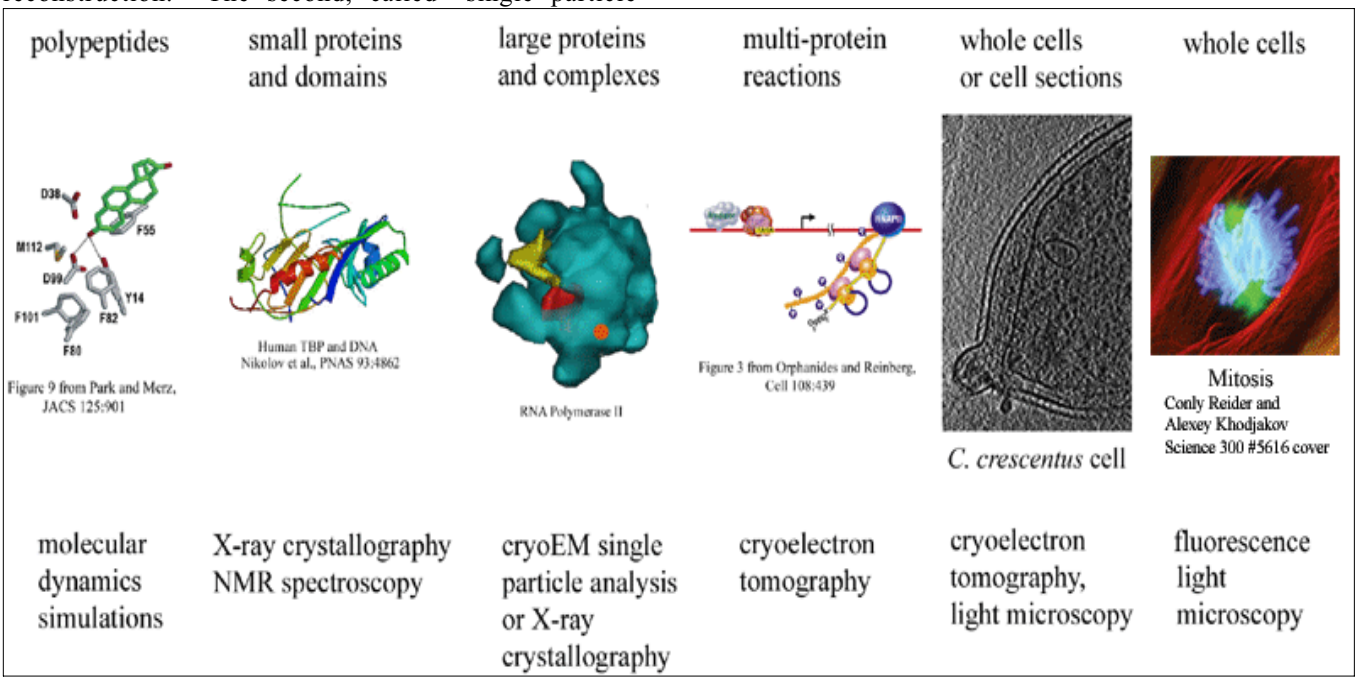
Summary: If we could simply look inside a cell and see its molecular components in all their complexes and conformations, cell biology would be all but finished. We are developing electron-cryomicroscopy-based technologies to do this for at least the largest macromolecular complexes, hoping to show both how individual proteins work together as large "machines" and how those machines are organized into "assembly lines" within living cells. Our electron cryomicroscopy projects range from imaging individual macromolecules to larger protein assemblies to viruses to organelles and even to intact cells. In addition, we have now begun simulation projects to understand how such spatial relationships affect cellular processes.

We use two basic data collection strategies. The first, called "tomography," involves imaging a single unique object (such as a cell) from multiple directions and then merging those projections into a three-dimensional reconstruction. The second, called "single particle analysis," involves imaging a large number of identical copies of a target object (such as a purifiable protein complex), and again merging the images to produce a three-dimensional reconstruction. Both techniques are still rapidly developing, but together hold the promise of completing what could be called the "structural biology continuum" (Figure 1) in a step-wise fashion by showing first how individual proteins (visible by X-ray crystallography and NMR spectroscopy) come together to form complexes (visible by single particle analysis), and how those complexes are organized within living cells (visible by electron tomography). This structural data, in turn, will inform whole-cell models of the interactions of all the cell's molecular components.

Both single particle analysis and tomography begin by spreading the sample in a thin film (~300 nm) across an electron microscope grid and then plunging it into liquid ethane, which causes the water to form vitreous, rather than crystalline, ice, and preserves the sample in a native state without any unnatural fixatives, resins, or stains. For tomography, the sample is imaged from a series of angles by incrementally tilting a goniometer through ~140 degrees. For single particle analysis, each copy of the sample (one "particle") freezes with a random orientation with respect to the plane of the grid so that tilting is not necessary. Instead, images of hundreds of thousands of individual particles are recorded and then aligned and averaged in three-dimensions computationally. The target resolution for single particle analysis is 4-10Å, where the secondary structure of a particle can be seen and fitting atomic models of components is possible. The target resolution for tomography is 3-6 nm, where the identity, location, and orientation of individual macromolecules can be seen in their cellular contexts.



Our work involves technology development as well as its application to key biological questions. We enjoy the use of a state-of-the-art, 300 kV, helium-cooled, energy-filtered, fully automated, dual-axis tilting, FEG "Polara" TEM. This year's technical efforts were directed towards (1) simulation studies to understand the effect of alignment errors in single particle reconstructions, (2) theoretical work on methods to correct for curvature of the Ewald sphere, and (3) developing software to automate tilt-series acquisition. Some of the biological questions we have addressed this year are (1) the nature of the prokaryotic cytoskeleton as seen by bioinformatic comparisons and tomograms of *Caulobacter crescentus, Magnetospirillum magneticum, Treponema primitia* and *Mycoplasma pneumoniae* cells; (2) the structure of the bacterial flagellar motor; (2) the structure of immature HIV-1 virus-like particles; (4) the quaternary structure of various RNA polymerase complexes; (5) the structure of the bacterial carboxysome; and (6) the ultrastructure of the smallest known eukaryotic cell.

247. Cryo-electron microscopy and single particle analysis

Andrew A. Rawlinson, Grant J. Jensen, J. Bernard Heymann

Single particle analysis techniques allow us to obtain three-dimensional reconstructions of frozen hydrated proteins by 'averaging' many thousands of electron microscope images of the protein. A crucial step in the process is the accurate determination of the alignment parameters – i.e., center, orientation and defocus – of each protein molecule/complex in the image – as inaccuracies (together with the effects of the contrast transfer function, Poisson (shot) noise, distortions due to microscope, etc.) can cause loss of high frequency information – thereby reducing the quality of the reconstructions.

The usual paradigm to overcome the loss of information is to collect more and more images, thereby improving the signal-to-noise ratio – but this depends on various factors, including the magnitudes of the alignment errors. Published in the literature are theoretical relations between the magnitudes of such errors and the number of images required to achieve a given resolution in a reconstruction. We are testing these relations by using simulated images (produced by Bsoft) and introducing alignment errors of varying magnitudes, so we can examine how many images are needed to achieve 'near atomic resolution' (~4 to 6 Å) reconstructions.

Using the Biology Division's supercomputer, we have created two 50,000-image databases, one database of the 20S proteasome, and the other of RNA polymerase. Alignment errors in each image are introduced and the quality of the resulting reconstructions is measured by comparing each reconstruction against a three-dimensional reference via the Fourier shell correlation function. Knowledge gained from these simulations can help us understand how robust orientation-finding programs (e.g., Bsoft) need to be in order to achieve near atomic resolution reconstructions of proteins when using real electron microscope images. Preliminary indications are that around 10,000s – 100,000s of images are required – something that is within the capability of current computer technology.

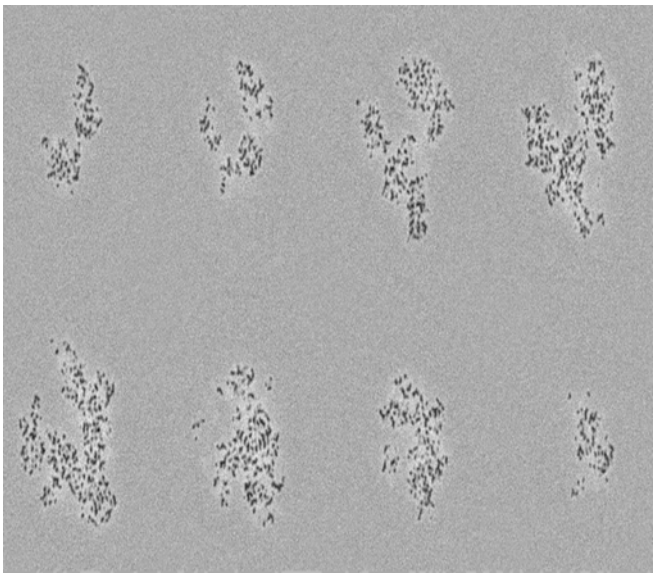


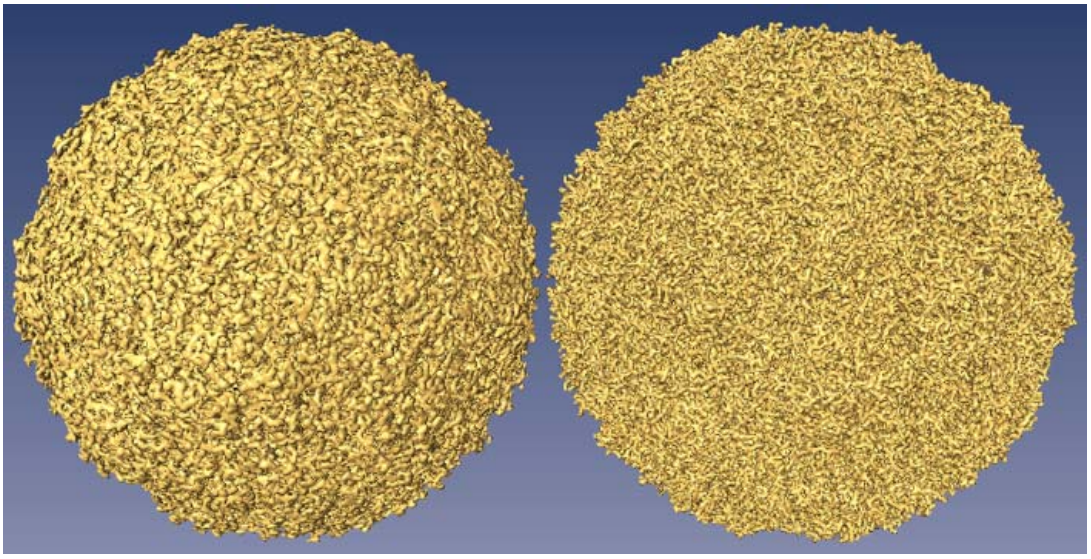
Figure: Cross sections of a 3D reconstruction of RNA polymerase using 50,000 simulated images.

248. Ewald sphere correction for large icosahedral virus particle reconstructions

Peter W. K. Leong, Grant J. Jensen

When an electron microscope records an image of an object, information from the Fourier values on the Ewald sphere contribute to form an image. The usual approximation made is that the image is a perfect projection of the object and instead contains information from a central slice of the 3D Fourier transform of the object. However, the approximation begins to fail at high resolutions, low acceleration voltages and for large virus particles because under these conditions, the information on the Ewald sphere becomes significantly different from the information found on a central slice, thereby limiting the maximum resolution of the 3D reconstruction of the object.

We have developed a reconstruction algorithm to take into account the curvature of the Ewald sphere and are implementing it in two different image-processing packages. Our simulations have shown that the resolution limit, which is a result of the central slice approximation, can be corrected for, thereby improving the resolution of 3D reconstructions. The algorithm is also being applied to an experimentally obtained dataset of a large virus particle to confirm the resolution improvements on real data.



4Å (left) and 2Å (right) reconstructions of Foot and Mouth Virus capsid using Ewald-sphere-simulated-images illustrate the improvement in resolution when taking the Ewald sphere into account.

249. Automatic multiple TEM tilt series acquisition using Leginon

Christian Suloway, Grant J. Jensen

Despite available data collection software, transmission electron microscope (TEM) tomography is still a labor-intensive technique requiring a user to spend hours or even days at the instrument. The problem arises from the need to not only tilt and track the specimen when imaging, as implemented in current software, but locate, position and focus multiple areas of interest. By integrating the predictive tracking algorithm and tilt series collection capability from the software application UCSFTomo[1] into a software package for automating data collection on TEMs, Leginon[2], the data collection process is now fully automated.

Many new features have been added to Leginon during the integration including general utilities for acquiring and storing tilt series data and optimization of the geometric model for change in position of the specimen in three dimensions as it is tilted. Additional database structures for storing tilt series and tracking information have been added. New web pages for viewing image data, analyzing specimen tracking algorithm performance, and accessing collected data have been added to the Leginon support tools.

The data acquisition workflow begins by Leginon collecting low magnification images of the specimen and stitching them together into an atlas. Areas of the atlas can then be selected in the user interface to be imaged at increasing magnifications, and this information is used to choose targets for tilt series collection. Leginon then proceeds from one target to the next, performing all of the tasks necessary for collecting high quality cryo-electron microscope tilt series data using an energy filter, including setting focus and eucentric height, realigning the energy filter slit, and recording full tilt series without ever needing human interaction. A drift monitoring system in Leginon ensures tilt series are taken at the correct locations on the specimen regardless of specimen drift over time. The current rate of data acquisition is roughly 30 seconds per tilt image on a 2048 pixel square CCD camera, comparable to UCSFTomo.

This software is expected to significantly increase productivity by facilitating routine 24-hour data collection, reducing the perturbations caused by a constant rotation of users and their individualized setup routines, and identifying microscope performance problems immediately.

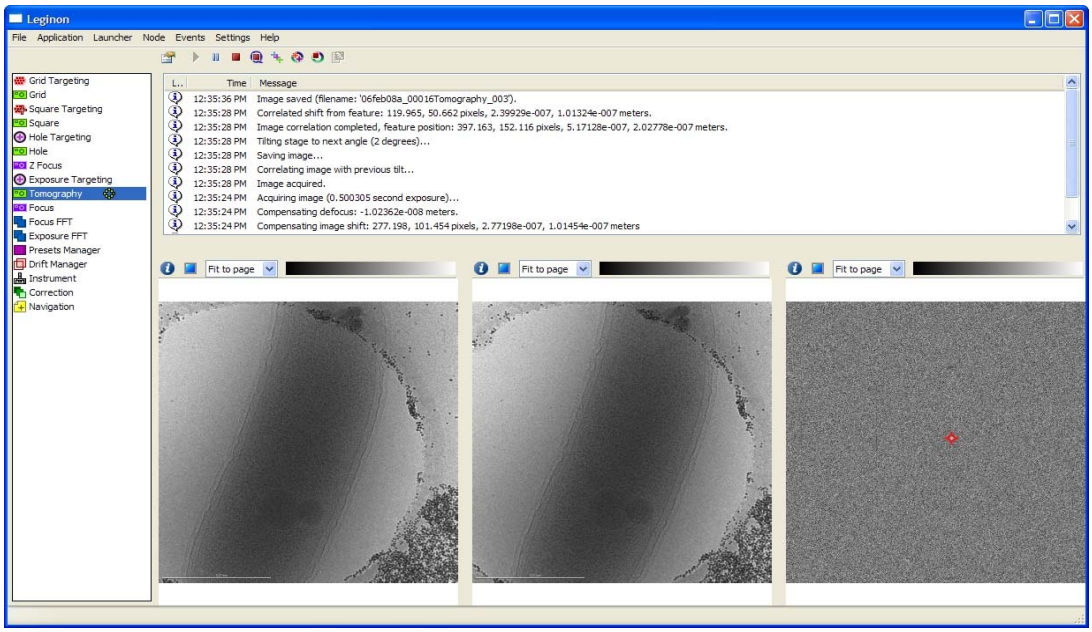


Figure 1. Screenshot of Leginon collecting a tilt series.

Figure 2. Screenshot of the tilt-series diagnostic web tool. Graphs show the predicted (blue), actual (orange) and relative positions (green) of the specimen in pixels per tilt angle in degrees for the x and y axes of the CCD camera.

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250. Online database of Prokaryote genotype-phenotype associations

Dylan Morris, Grant J. Jensen

Gene phylogenetic profiles have become a powerful tool for comparative genome analysis. For a given set of sequenced genomes, the phylogenetic profile of a particular gene is a vector of ones and zeros that indicate whether homologues of that gene are present or absent in each of these genomes. These profiles have traditionally been used to assign functions to newly sequenced genes based on their statistical relationships to the profiles of genes of known function. In a recent paper, Slonim *et al*., introduced a method for using phylogenetic profiles to probe correlations between genes and behavioral phenotypes. Specifically, they used mutual information analysis to determine those genes whose presence in a microbial genome is highly predictive of that organism exhibiting a particular phenotype. They performed this analysis and a subsequent aggregation step to arrive at a set of generic gene modules that give high information about each of the examined phenotypes.

We are currently extending this work by creating a comprehensive, machine-readable database of all microbial organisms whose genomes have been completely sequenced. This database will contain phylogenetic profiles for every gene in each of these genomes and will characterize the organisms across a wide range of phenotypes. We are constructing the phenotype database by manually mining the published research literature. We will use these data to search for sets of phenotype-specific genes (genes that are highly conserved across those microbial organisms that display the phenotype and absent from those that do not) across the entire range of sequenced microbial genomes. At present, there are 354 completed microbial genomes available on the NCBI server, and an additional 594 are currently in progress.

As newly completed genomes are added to the NCBI database, our software will automatically build the corresponding set of phylogenetic profiles and incorporate them into our database. The software will periodically recalculate the global genotype-phenotype relationships in order to include newly added genomes in the analysis. The results will be presented on an interactive website, where users will be encouraged to add new phenotype information to the database and will be allowed to run the genotype-phenotype correlation analysis against their own data.

251. Image processing software tool development and applications

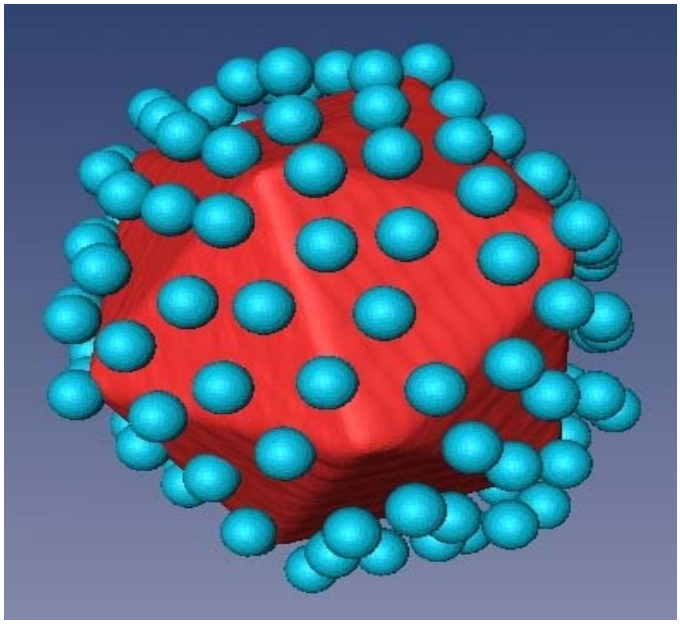
H. Jane Ding, Grant J. Jensen

Today's electron microscopy is strongly dependent on software development. Software in image processing plays crucial roles in the field. From single particle reconstruction to tomography, we use various software packages for reconstructions and structural analysis. We employ existing software packages as well as write our own packages as needed. We have been developing various techniques in tomogram analysis for obtaining structural information of the molecular complexes through electron microscopy, for example, building modules that project layers of density maps to surfaces so a better view of the layer structure of object can be obtained. Below are some of the major projects we are currently working on:

- Tomogram segmentation: Manual segmentation of tomograms is often subjective and can be tedious. We are working on algorithms for quantitative approaches to the interpretation of features in tomograms, either automatically or semi-automatically.
- Tomogram search: Computationally search the three-dimensional reconstructions of cells to identify macromolecular complexes and cytoskeletal elements of interests.

Tomogram analysis is challenging because of the low signal-to-noise ratios of the EM images as well as the "missing wedge" in tomography. Our future work is also aiming at improving the quality of the images, which includes implementing new software or improving the software that is currently used in three-dimensional reconstruction in tomography, especially for "dual-tilt" tomography.

The figure below is an image of an icosahedral layer of rubisco molecules inside of a carboxysome. Computed and generated from actual EM data by various software tools.



252. Electron-cryo-tomography of the prokaryotic cytoskeleton in *C. crescentus* mutants

Ariane Briegel, D. Prabha Dias, Zhuo Li, Lu Gan, Mike Trimble[*], Yves Brun[*], Christine Jacobs Wagner[†], Zemer Gitai[§], James W. Gober[‡], Grant J. Jensen

Until relatively recently, it was thought that bacteria had very little regular internal structure and that they were essentially a "bag of macromolecules." There is now mounting evidence that this is not the case, including the discovery of FtsZ and MreB, the bacterial homologs to the eukaryotic cytoskeletal proteins tubulin and actin, respectively, and the visualization by electron-cryo-tomography of filaments in bacteria. The sequencing of the *C. crescentus* genome revealed the presence of cresentin, a homolog to a third type of cytoskeletal element, intermediate filaments. This bacterium is thus an excellent place to look for a bacterial version of the eukaryotic cytoskeleton. Last year, we successfully visualized several types of filaments in wild-type *C. crescentus.*

This year, we have obtained three-dimensional structures of several types of mutant *C. crescentus* with the purpose of identifying the proteins that make up each of the filaments that we have seen. These mutants include one in which FtsZ hydrolysis has been blocked and which exhibited elongated constriction sites containing large numbers of filaments partially circling the cell, perpendicular to the long axis (see figure).

This result is compelling evidence that similar filaments seen in wild-type cells are composed of FtsZ. Strains with mutations for crescentin overexpression, crescentin deletion and non-functional crescentin are similarly helping to identify which wild-type filament is composed of crescentin, although the results are not as clear-cut as in the FtsZ case. Mutants with MreB overexpression and ParA overexpression are also being studied by electron-cryo-tomography.

The ParA gene product is essential for chromosome segregation in *C. crescentus* and *B. subtilis* and has been localized to a transient filamentous structure by fluorescent light microscopy (fLM). We are also interested in the related topic of general cell shape determination in Caulobacter, and to that end are studying mutants that start bleeding when depleted of a cell envelope regulator kinase. These blebs appear to be formed by the separation of the outer membrane from the inner membrane.

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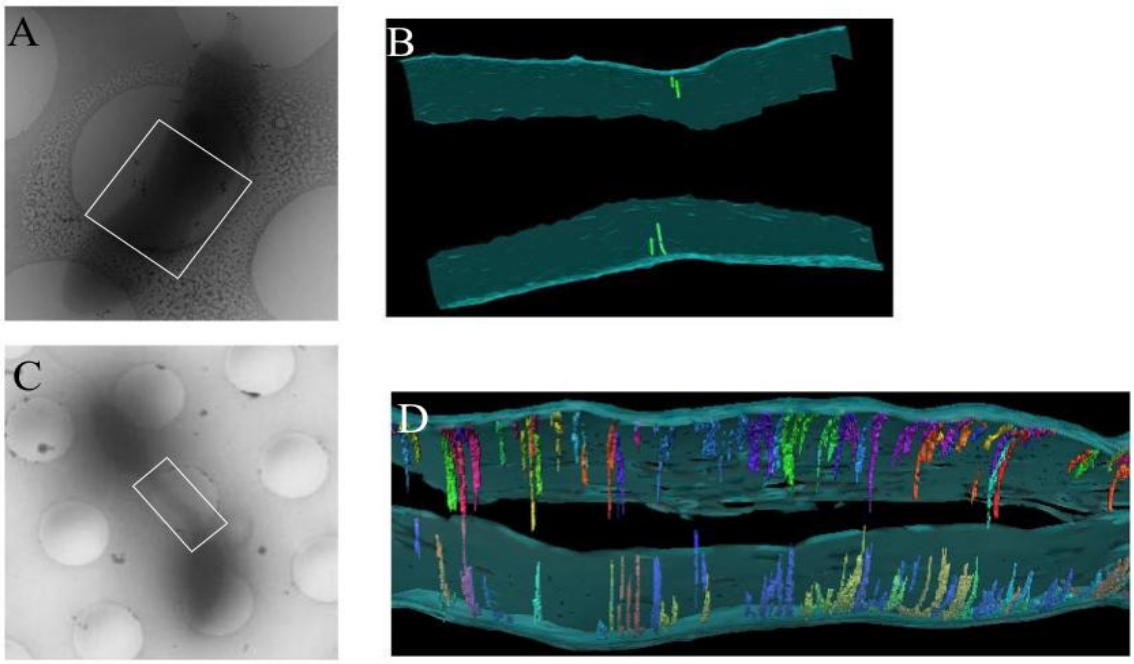


Figure 1. FtsZ rings in *Caulobacter crescentus.* A dividing wild-type cell (*A, B*) and an FtsZ mutant cell (*C, D*) were flash-frozen in vitreous ice and imaged by electron cryotomography. (*A*) and (*C*) are snapshots after tilt series collection. (*B*) and (*D*) show segmentations from tomograms of regions indicated by the white boxes in (*A*) and (*C*), respectively. The width of wild-type *Caulobacter crescentus* is ~500nm. The extended area in the mutant cell has a width of ~ 300 nm.

253. Correlated fluorescent light microscopy and electron-cryo-tomography of bacteria

Ariane Briegel, Rasmus Bugge Jensen, Grant J. Jensen*

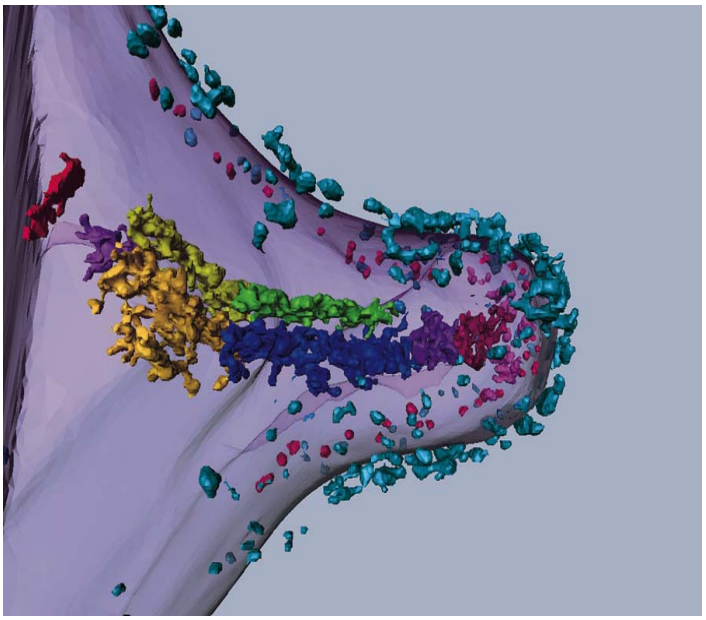
One of the few disadvantages of electron-cryo-tomography (ECT) when compared with fluorescent light microscopy (fLM) is that there is currently no way to directly label a protein for ECT in the same way that GFP can be used to label proteins for fLM. The only labeling methods in use for ECT are immunology-based, and thus of limited accuracy and scope. Another disadvantage of ECT versus fLM is that fLM can capture dynamic processes, while ECT is necessarily a static image of the specimen (it is frozen, after all). However, the huge advantage of ECT over fLM is its much higher resolution and, in the case of conventional fLM, the three-dimensional nature of ECT structural data. To take advantage of the best of both techniques, we are developing a method for correlated fLM and ECT of bacteria. The concept is to obtain an image of a bacterium in the fLM and then image the same exact bacterium by ECT. We have so far succeeded in immobilizing GFP-labeled *Caulobacter crescentus* on EM grids, then placing these grids on glass slides and imaging them in the fLM at both low and high magnifications. We also successfully plunge-froze the grid with the imaged bacteria and were able to find the same bacterium and image it in our FEI Polara microscope. So far the fLM-imaged cells appear to be distressed compared to cells that have gone from culture straight to being plunge-frozen. Current efforts are directed at optimizing the process to reduce the time needed for fLM imaging and to introduce other measures to reduce the stress on the cells.

**Department of Microbiology, Roskilde University, Roskilde, Denmark*

254. The structure of *Mycoplasma*'s attachment organelle and its role in motility

Gregory Henderson, Grant J. Jensen

While most motile bacteria propel themselves with flagella, other mechanisms have been described including retraction of surface-attached pili, secretion of polysaccharides, or movement of motors along surface protein tracks. These have been referred to collectively as forms of "gliding" motility. Despite being simultaneously one of the smallest and simplest of all known cells, *Mycoplasma pneumoniae* builds a surprisingly large and complex cell extension known as the attachment organelle that enables it to glide. Previously, three-dimensional images of the attachment organelle were produced with unprecedented clarity and authenticity using state-of-the-art electron cryotomography. The attachment organelle was seen to contain a multi-subunit, jointed, dynamic motor much larger than a flagellar basal body and comparable in complexity. A new model for its function was proposed wherein inchworm-like conformational changes of its electron-dense core are leveraged against a cytoplasmic anchor and transmitted to the surface through layered adhesion proteins.[1]



Above: Cover photo, *Molecular Microbiology*

This model is currently being tested with further work on *Mycoplasma pneumoniae* and its mutants.

Publication

1. Henderson, G.P. and Jensen, G.J. (2006) Three-dimensional structure of *Mycoplasma pneumoniae's* attachment organelle and a model for its role in gliding motility. *Mol. Microbiol*. **60**(2):376-385.

255. *In situ* structure of the complete *Treponema primitia* periplasmic flagellar motor

Gavin Murphy, Jared Leadbetter, Grant J. Jensen*

I generated a 7 nm resolution, 3-D average of an *in situ* flagellar motor. The elusive stators are visible for the first time and their natural interactions with other components and the membrane are preserved. This work advances our structural understanding of the flagellar motor, much of which focused on the purified basal body, which lacked the stators. The flagellar motor is an amazing nanomachine built from many different proteins and uses an electrochemical ion gradient to drive rotation at speeds up to 300 Hz in some organisms. The motor consists of a fixed, membrane-embedded, torque-generating stator and a typically bidirectional, spinning rotor that changes direction in response to chemotactic signals. Stator studs extend into the periplasm and grip the peptidoglycan layer (PG) for rigidity, while the cytoplasmic stator portion spins a wheel, the C ring, and its attached hub, the rotor. The flagella rod rises from the rotor through a proteinaceous P ring "bushing" in the PG.

The final structure shows the stator studs extending above the membrane and leaning CCW (dotted line in Figure 1a, below). The center-to-center diameter of the stator studs is 61 nm. The stator is embedded in the inner membrane (IM) and contacts the C ring through thin bridging density (connection #1 in Figure 1b), and

unexpectedly, it also contacts the rotor in two places (#2 and #3) and a P-ring-like shape labeled the P collar (#4). The stator studs (asterisked in 1d) are 8 nm wide and thin-bridging density connects them (arrowhead). A pore shape lies on the rotor's bottom (arrow in 1e), with an "export bundle" shape beneath it. The PG and outer membrane (OM) are not visible in the averaged structure but are pictured in their expected locations (Figure 1c). It is thought that the torque-generating interaction occurs at connection #1.

These results highlight the ability of cryoelectron tomography to not only reconstruct the 3D structures of individual macromolecular complexes but to also average them for increased resolution.

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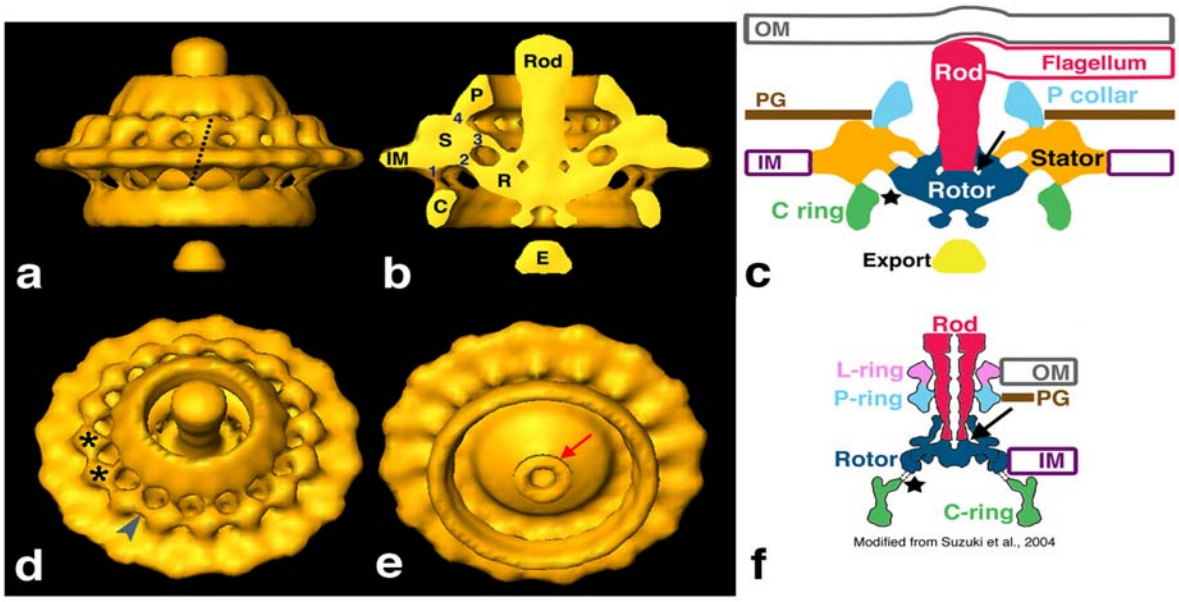


Figure 1: The *Treponema primitia* periplasmic flagellar motor. a,b,d,e: Isosurfaces of the *T. primitia* motor, with components marked in **b**. **c:** Cartoon of the *T.p.* motor with labeled components. **f:** Cartoon of the isolated *Salmonella* basal body with labeled components.

256. Electron cryotomography of the CA and SP1 domains of immature HIV-1 gag

Elizabeth R. Wright, Jordan Benjamin, H. Jane Ding, Grant J. Jensen

The major structural elements of retroviruses are contained in a single polyprotein, Gag, which in HIV-1 comprises the matrix (MA), capsid (CA), SP1, nucleocapsid (NC), SP2, and p6 proteins. In the immature HIV-1 virion, the domains of Gag are arranged radially with the amino-terminus of MA at the membrane and NC-p6 facing the particle center. While assembly of the mature virion from the immature form has been studied extensively, and the structural elements of both have been identified and determined, a complete understanding of the immature virion has remained elusive. Here, we report the first three-dimensional structure of individual immature HIV-1 virions obtained by electron cryotomography. We measured the virions and found the diameters to range from 115 to 140 nm with a mean of 127 nm. The radial spoke arrangement of the Gag polyprotein was clearly visible in the tomograms. The thicknesses of the domains of the Gag polyprotein were measured as 78 ± 10 Å for membrane-MA, 77 ± 10 Å for CA, and 43 ± 10 Å for NC. Upon producing surface projections of each layer of Gag, it was determined that only the CA domains contained regions of order within the virions (Figure 1).

The CA domains were arranged in hexagonal lattices interspersed between patches of disorder. We developed a novel method for detection and averaging of unit cells from highly ordered regions within the Gag shell. A three-dimensional map produced by averaging 120 unit cells revealed a two-layer hexagonal lattice in the CA and SP1 domains. The ring-to-ring spacing of the CA NTD lattice was 8 nm and the ring-to-ring spacing of the SP1 lattice was 7.5 nm. From our data, we describe a pseudoatomic model of the CA and SP1 domains of immature HIV-1 in which SP1 hexamers appear to stabilize individual CA hexamers (Figure 2).

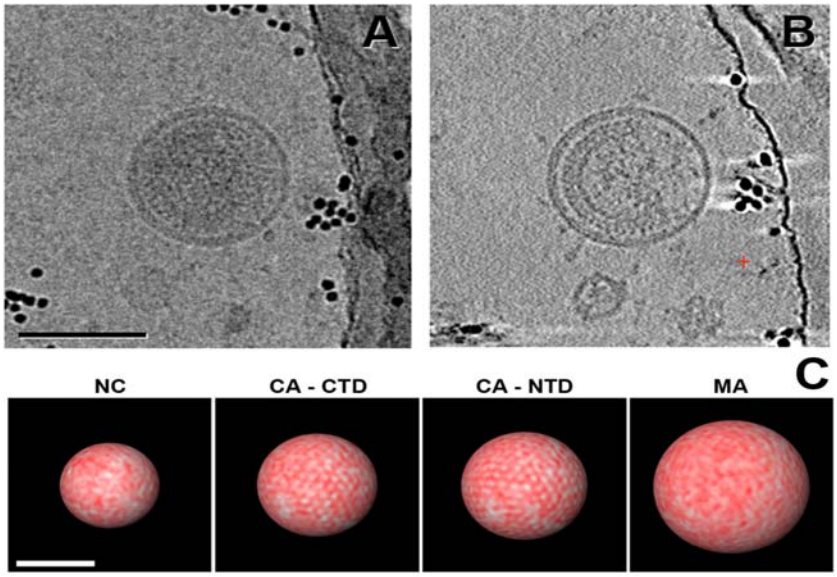


Figure 1. Isolated HIV-1 virion preserved by flash freezing and imaged by cryo-ET. (A) The central image from a tilt series of an immature HIV-1 virion VLP. (B) A 5.6 nm slice trough the middle of the reconstruction depicting the same area as in A. (C) One representative immature virion displaying the protein densities associated with each layer of the Gag polyprotein (left to right: NC, CA CTD, CA NTD, MA). In the surface projections, it was observed that only the CA layers contained regular patterning attributed to the protein densities. Red is more dense. Scale bar for (A) and (B) 100 nm, scale bar for (C) 50 nm.

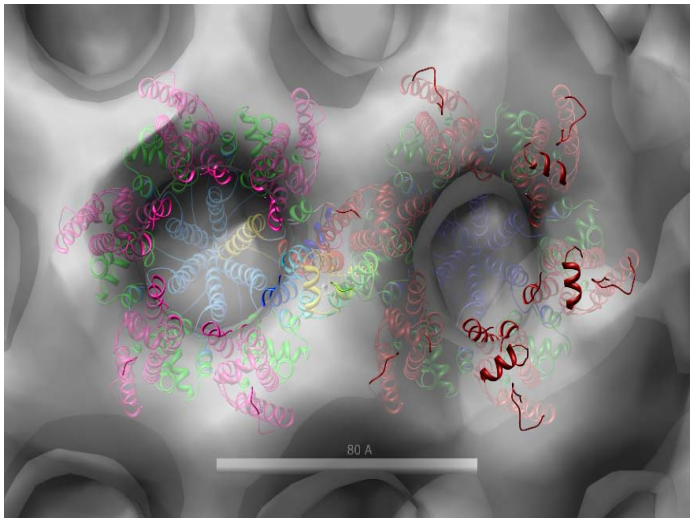


Figure 2. Top view of immature HIV-1 Gag components docked into the averaged density. The color code is as follows: CA NTD is pink (left) or red (right), CA CTD is green (both), and SP1 is light blue (l) or purple (r). Scale bar is 80 Å.

257. Imaging transcription complexes using cryoelectron microscopy

Zhiheng Yu, Grant J. Jensen

Transcription is one of the most complicated cellular processes with many proteins participating at various stages to regulate gene expression. In eukaryotes, for a TF to fulfill its biological function of transcribing certain protein-encoding genes, it usually needs to interact with RNA polymerase II (RNAP II) basal transcription complexes. It is critical to pursue the structural details of the RNAP II transcription complexes in order to understand transcription in general and mechanisms leading to specific diseases in particular.

The two transcription complexes studied in this work are PTB and PFB, respectively. The former is composed of RNAP II, TATA-box binding protein (TBP), basal transcription factor IIB (TFIIB) and a 41mer-5mer DNA-RNA hybrid and the latter is composed of RNAP II, TFIIB, TFIIF and a 53mer-7mer DNA-RNA hybrid. The significance of these complexes is that the complexes are captured in an early "transcribing" state.

Electron tomography was performed on ~150 copies of the PTB complexes suspended in thin vitrified ice. The projected images in the tilt series were back projected and processed to generate three-dimensional (3D) reconstruction. From the 3D reconstruction, I boxed out 80 copies of the PTB complexes, aligned them and averaged them to generate a low-resolution 3D model for the PTB particle. Figure 1a shows the averaged 3D model in which the main features can be clearly seen. Considering the relatively small molecular weight of the transcription complexes (600-700 kDa), it is encouraging to see that electron tomography can produce a 3D model of such quality. This model will serve as the initial model for the higher-resolution single particle analysis. I have already collected hundreds of high-resolution projection images of PTB and PFB particles suspended in very thin ice at random orientations with an optimized dose and at various defocus values. A first round single-particle reconstruction using the software EMAN was carried out for PTB and a similar process for PFB is currently under way. Figure 1(B) shows the difference map of the reconstructed PTB relative to the crystal structure (PDB ID: 1SFO) of RNAP II with a 15mer- 9mer DNA-RNA hybrid. We can clearly see the dumbbell shaped c-TFIIB located at almost the same location as in the co-crystal of RNAPII and TFIIB (PDB ID: 1R5U).

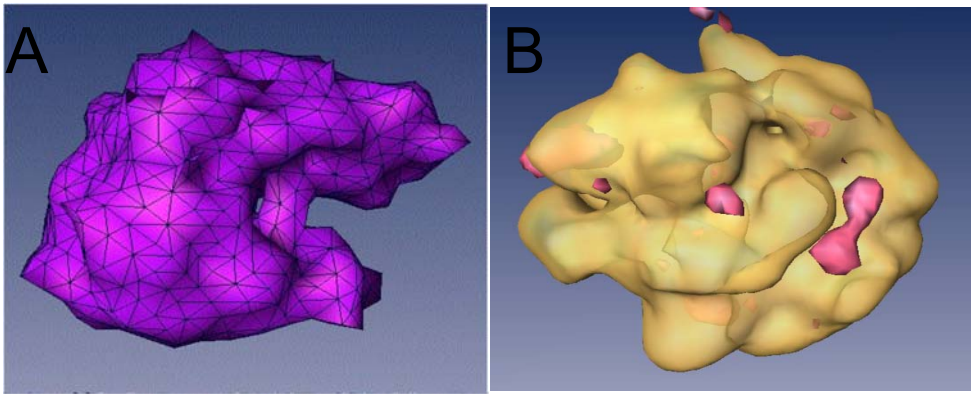


Figure 1: (**A**) Electron tomography reconstruction of PTB. This model was generated by averaging 80 particles from a cryoelectron tomogram. (**B**) Difference map (in red) of single particle analysis reconstruction of PTB with respect to the crystal structure of RNAPII with 15mer-9mer DNA-RNA hybrid (PDB ID: 1SFO). The difference map is rendered at three standard deviation and the electron density of 1SFO is shown in transparent yellow.

258. Electron cryotomography of purified carboxysomes

Cristina V. Iancu, H. Jane Ding, Dylan M. Morris, Grant J. J. Jensen

Carboxysomes are organelle-like polyhedral bodies enclosed by a proteinaceous shell and filled with ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO) molecules, a key enzyme in CO_2 fixation. They are part of the CO_2 concentrating machinery (CCM) in cyanobacteria and many chemoautotrophic bacteria, and are produced in a greater number when the organisms are grown under low-CO_2 conditions. Exactly how carboxysomes help the organisms compete when CO_2 is limited is still unclear. We employ electron cryotomography to explore the 3D structure of purified carboxysomes. These studies will provide a more detailed view of these organelles allowing us to determine their overall shape, the number and organization of RuBisCOs, thus leading to a better understanding of the structure-function relationship.

We have looked at carboxysomes purified from two different sources: the *Synechococcus* strain WH8102 and *Halothiobacillus neapolitanus (H. neapolitanus)*. Both kinds of carboxysomes vary in size and they have an icosahedral overall shape that seems more regular for *Synechococcus*. RuBisCO molecules are organized in shells spaced about 12 nm apart. Simulations of carboxysomes in which RuBisCO molecules are randomly placed revealed that, as the number of enzymes increased, shells spontaneously arise similarly to the ones in the data.

So far the resolution of the data is insufficient to support specific interactions between RuBisCOs. Recent studies on the shell proteins showed that two of these proteins form charged pores that probably provide charged metabolites to the RuBisCOs, and another shell protein is a novel carbonic anhydrase supposed to concentrate CO_2 proximally to RuBisCOs. Thus it appears that the carboxysome shell acts both as a permeable membrane for substrates and as a template, organizing RuBisCO in specific patterns that optimize CO_2 fixation.

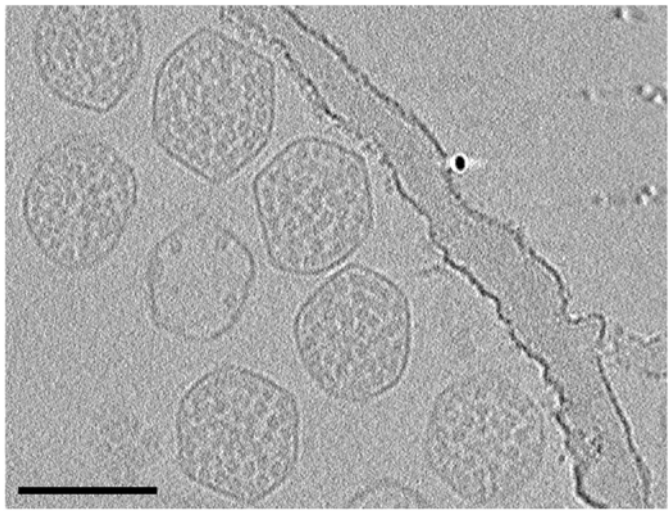


Figure: Slice through a tomogram of purified *H. neapolitanus* carboxysomes. Scale bar corresponds to 150 nm.

259. Electron cryotomography of *in situ* carboxysomes

Cristina V. Iancu, Grant J. Jensen

In parallel with the studies of purified carboxysomes, we also examine carboxysomes within cells to ensure that the purified material is representative for the *in vivo* carboxysomes and to establish what cellular ultra-structural elements are associated with them. A long-term goal is to capture different stages of carboxysome assembly within cells.

As *Synechococcus* cells are too large to be amenable to electron cryotomography studies we chose *H. neapolitanus* cells for visualizing carboxysomes *in situ*. Tomograms of these cells typically show about seven carboxysomes/cell. Most cellular carboxysomes are similar in size and shape to the purified ones, however, there are also some unusually long. There are no visible filaments linking the carboxysomes, they just seem to be dispersed randomly in the cytoplasm of the cell. The most surprising finding is the existence of a high-density aggregate of variable size within the majority of the cellular carboxysomes. As this element has never been seen in the purified carboxysomes we speculated that it might be the result of accumulated metabolites that have not been released yet from carboxysomes and that the purification process would dilute them away. We are in the process of testing this hypothesis by looking at cells that are slowed down metabolically.

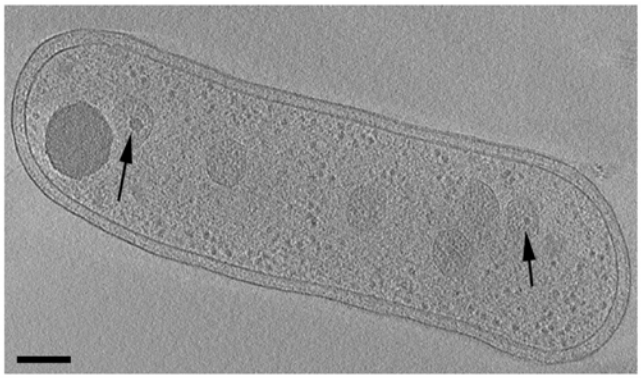
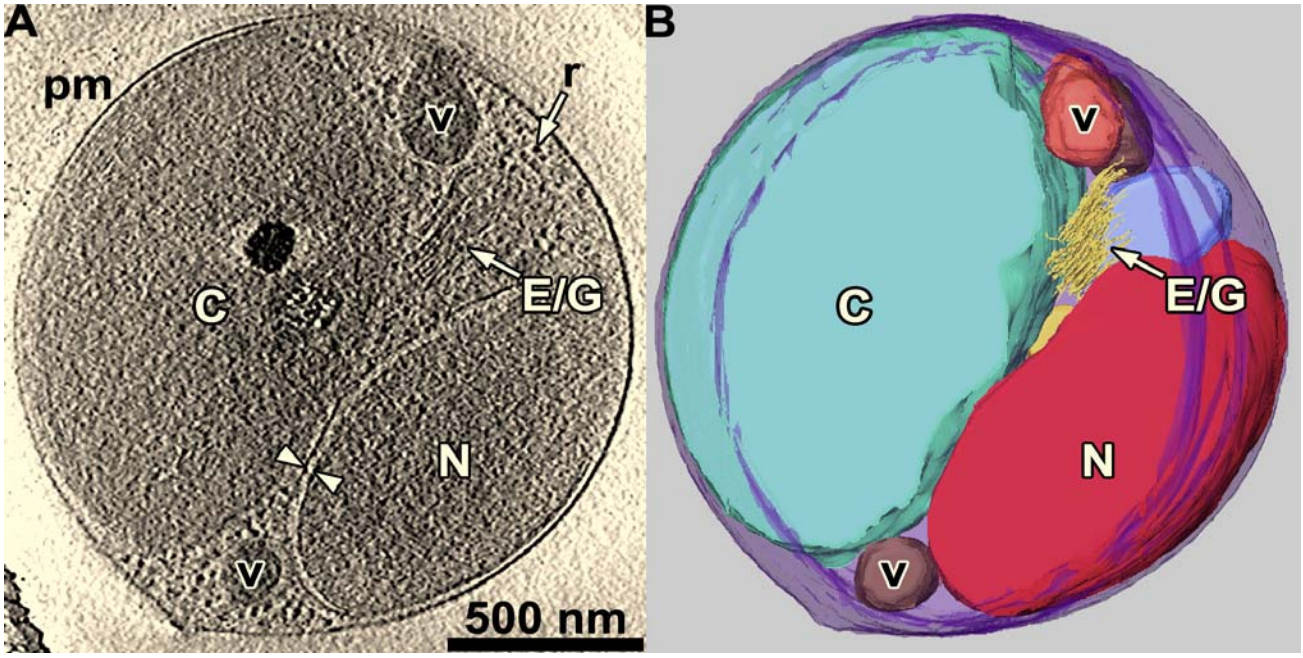


Figure: Slice through a tomogram of an *H. neapolitanus* cell. Scale bar corresponds to 150 nm. Arrows indicate the high-density aggregates present in cellular carboxysomes.

260. Electron cryotomography of the minimal eukaryote, *Ostreococcus tauri*

Gregory Henderson, Lu Gan, Grant J. Jensen

The picoplankton *Ostreococcus tauri* is the smallest known eukaryote. The 12.6 MB genome of *O. tauri* has just 5,441 nuclear genes, but includes a minimal-yet-complete set of cell-cycle control genes. This photoautotrophic unicellular organism, which measures 1 – 2 μm in diameter, is also the simplest of eukaryotes: it has just one nucleus, one chloroplast, and one mitochondrion. These properties make *O. tauri*, to our knowledge, one of the few eukaryotes that can be imaged *in toto* by electron cryomicroscopy. We have used electron cryotomography to determine the first life-like structures of whole, vitrified *O. tauri* cells. We confirmed earlier findings that each cell has one copy of each the organelles listed above, and we also found that each cell has a number of storage vesicles, and a Golgi body that is adjacent to the endoplasmic reticulum (ER). The remaining volume within the cytosol is packed with dense, ribosome-like particles. The chloroplasts, mitochondria, and storage vesicles have consistent shapes in most of the cells we have analyzed. By contrast, the nuclei are pleiomorphic. Most of the cells that we have examined have an "open" nucleus, indicating that *O. tauri* undergo open mitosis, which is characterized by either partial or full nuclear-envelope breakdown. The nucleus is simultaneously adjacent to the ER, the chloroplast, the mitochondrion, and the plasma membrane; this is a consequence of the small cell-size. The lumen, which separates the inner- and outer-nuclear envelope, has uniform width when the outer envelope is in contact with another organelle, but variable width when it faces the cytosol. Our tomograms of *O. tauri* provide authentic 3-D views of eukaryotic ultrastructure.



A) Tomographic XY-slice (3.6-nm thick) through the center of an *O. tauri* cell. (B) three-dimensional, volume rendering of partially segmented cell from (A). Tentative assignments of structures are abbreviated: g, granule; r, ribosome; v, vesicle; pm, plasma membrane; N, nucleus; C, chloroplast; E/G, endoplasmic reticulum / Golgi body. Nuclear inner- and outer-envelopes denoted by two white arrowheads.

Publications

Briegel, A., Dias, D.P., Li, Z., Jensen, R.B.J., Frangakis, A.S. and Jensen, G.J. (2006) Multiple large filament bundles observed in *Caulobacter crescentus* by electron cryotomography. *Mol. Microbiol.* Submitted for publication.

Henderson, G.P. and Jensen, G.J. (2006) Three-dimensional structure of *Mycoplasma pneumoniae's* attachment organelle and a model for its role in gliding motility. *Mol. Microbiol.* **60**(2):376-385.

Iancu, C.V., Wright, E.R., Heymann, J.B. and Jensen, G.J. (2006) A comparison of liquid nitrogen and liquid helium as cryogens for electron cryotomography. *J. Struct. Biol.* **153**:321-240.

Komeili, A., Li, Z., Newman, D.K. and Jensen, G.J. (2006) Magnetosomes are cell membrane invaginations organized by the Actin-like protein MamK. *Science* **311**:242-246.

Murphy, G.E. and Jensen, G.J. (2005) Electron Cryotomography of the *E. coli* pyruvate and 2-oxoglutarate dehydrogenase complexes. *Structure* **13**:1765-1773.

Murphy, G.E., Leadbetter, J.R. and Jensen, G.J. (2006) *In situ* structure of the complete *Treponema primitia* flagellar motor. *Nature*. Submitted for publication.

Wright, E.R., Iancu, C.V., Tivol, W.F. and Jensen, G.J. (2006) Observations on the behavior of vitreous ice at ~82 and ~12K. *J. Struct. Biol.* **153**:241-252.

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Research Fellows: Karin Crowhurst, Possu Huang, Alex M. Perryman, Thomas P. Treynor
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Research and Laboratory Staff: Marie L. Ary, Cynthia L. Carlson, Rhonda K. Digiusto
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Summary: The focus of the lab has been the coupling of theoretical, computational, and experimental approaches for the study of structural biology. In particular, we have placed a major emphasis on developing quantitative methods for protein design with the goal of developing a fully systematic design strategy that we call "protein design automation." Our design approach has been captured in a suite of software programs called ORBIT (Optimization of Rotamers By Iterative Techniques) and has been applied to a variety of problems ranging from protein fold stabilization to enzyme design.

261. Efficient optimization algorithms for multi-state protein design

Benjamin D. Allen[1], Stephen L. Mayo

Traditional protein design methods select amino acid sequences that are consistent with a single, fixed, main-chain structure by optimizing the identities and conformations of the side chains. However, real proteins assume an ensemble of conformational states, and modeling sequences on a single backbone restricts our ability to select sequences that reflect this essential characteristic. Similarly, it is difficult to generate sequences with altered catalytic or binding specificity when only one state is modeled at a time. These limitations of single-state design have prompted interest in multi-state design (MSD) algorithms, such as multi-state design Monte Carlo (MSD-MC). Previous results have indicated that the Fast and Accurate Side-Chain Topology and Energy Refinement (FASTER) algorithm is significantly more efficient than Monte Carlo for single-state design. We developed and implemented a new optimization algorithm for multi-state protein design based on FASTER, and compared its performance to MSD-MC. Application of the MSD algorithms to several difficult single-state design problems revealed that MSD-FASTER was always able to find the optimal solution (as determined using well-tested single-state design algorithms), whereas the best solutions found by MSD-MC were sub-optimal. Both algorithms were able to successfully optimize the straightforward core design of a 60-member NMR ensemble. Although neither method was able to thoroughly sample a difficult surface design of the same ensemble in a reasonable time frame, the solutions produced by MSD-FASTER were significantly better. Finally, both algorithms were applied to a negative design problem with three states, and both were able to find an optimal sequence that stabilized one structure and destabilized the other two, as desired. The performance of MSD-FASTER was nearly 100-fold better than MSD-MC in this case. We conclude that MSD-FASTER is more efficient than MSD-MC for multi-state design in a variety of contexts.

[1]*Division of Chemistry and Chemical Engineering, Caltech*

262. Using negative design to increase HIV protease specificity

Oscar Alvizo[1], Stephen L. Mayo

HIV protease is essential for viral maturation. Its main role is to recognize and cleave sequences in the Gag and Pol polyproteins. The enzyme is of unique interest because its symmetrical binding region recognizes and cleaves asymmetrical substrates. In addition, its natural substrates exhibit little sequence homology (three are shown in the table below). Our goal is to exploit HIV protease's asymmetric substrate recognition by designing hetero-dimer mutants with increased specificity for one of three natural substrates, CA-p2, p2-NC or RT-RH. Crystal structures are available for an inactive variant of HIV protease bound to each of these substrates; these complexes serve as scaffolds for computational protein design.

The optimization procedure uses all three scaffolds simultaneously and employs a scoring function chosen to predict mutations that will stabilize one scaffold over the other two. To insure viable sequences, only sequences with energies within 20% of the global minimum energy conformation (GMEC) of the positive design structure are evaluated. Within this restraint, the search algorithm tries to recover sequences with detrimental interactions in the two negative design structures. The predicted mutants are visually inspected and promising mutations are selected for experimental validation.

This type of negative design procedure proved to be the most effective in producing desirable mutants. To date, three mutants, one for each of the substrates, have been selected and experimentally tested. Preliminary data suggest that each of these mutants exhibits increased specificity towards its intended substrate. More rigorous testing is currently underway to quantitate this effect. Subsequent rounds of negative design calculations are expected to further increase the specificity of the mutants towards their intended substrates.

[1]*Graduate Option in Biochemistry and Molecular Biophysics, Caltech*

Substrate	P5	P4	P3	P2	P1	P1′	P2′	P3′	P4′	P5′
CA-p2	Lys	Ala	Arg	Val	Leu	Ala	Glu	Ala	Met	Ser
p2-NC	Pro	Ala	Thr	Ile	Met	Met	Gln	Arg	Gly	Asn
RT-RH	Gly	Ala	Glu	Thr	Phe	Tyr	Val	Asp	Gly	Ala

263. Designing nonspecific lipid transfer proteins for biosensor applications

Eun Jung Choi, Jessica Mao*[1], Stephen L. Mayo*

Biosensors are biological molecules that recognize specific ligands and relay the message to an easily measured physical signal. Recently, bacterial periplasmic binding proteins (PBPs) with different ligand specificities were tagged with a fluorophore to produce biosensors for their natural ligands or for novel ligands [1]. The natural ligands of PBPs are hydrophilic and relatively small. The binding site residues are also hydrophilic, thus making it difficult to design in large hydrophobic ligands. Our aim is to expand the chemical scope of target ligands by developing a protein platform capable of specifically binding large and/or hydrophobic ligands. For this purpose, we selected nonspecific lipid transfer protein (LTP) from maize (mLTP).

mLTP contains four disulfide bridges: C4-C52, C14-C29, C30-C75, and C50-C89. Our strategy was to use the ORBIT protein design software to design out the disulfide bonds and thus allow flexibility in the mLTP molecule. ORBIT predicted five variants: C4H/C52A/N55E, C4Q/C52A/N55S, C14A/C29S, C30A/C75A and C50A/C89E. Circular dichroism wavelength scans indicated that three of the five variants (C4H/C52A/N55E, C4Q/C52A/N55S and C50A/C89E) were properly folded. Thermal stability studies in the absence and presence of palmitate showed that C4H/C52A/N55E and C4Q/C52A/N55S gained the most stability upon binding palmitate, suggesting that these mutants might undergo a large conformational change upon ligand binding. Therefore, they were predicted to be good candidates for biosensor design.

We mutated one of the original cysteine residues back in two of the variants, C4H/C52A/N55E and C50A/C89E. Acrylodan (Ac), an environment sensitive thiol-reactive fluorophore, was then conjugated to the resulting free cysteine in each protein. We titrated the protein-acrylodan conjugates with palmitate to test the ability of the engineered mLTPs to act as biosensors. C52A/C4-Ac and C4H/N55E/C52-Ac showed the most marked difference in signal when palmitate was added. We determined the Kd of palmitate to be 0.6 mM for C52A/C4-Ac and 4 μM for C4H/N55E/C52-Ac. These values were close to the 3 μM Kd of palmitate that we had obtained for wild-type LTP using tyrosine fluorescence.

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264. Structural and dynamic analysis of computationally designed protein G variants

Karin Crowhurst, Stephen L. Mayo

Protein function is frequently reliant on dynamic processes; successful protein-protein interactions and enzyme catalysis often require structural flexibility and plasticity. These entropic factors can also contribute to protein stability. Understanding dynamic motions in computationally designed proteins compared to wild type could therefore be crucial for improving the accuracy and consistency of stable *de novo* protein and enzyme production, with wide-ranging medical and industrial applications. My current project goal is to understand the degree to which our current protein design algorithm (ORBIT) produces proteins with native-like backbone dynamics; information learned can be used to target future algorithm modifications and to move towards deliberately incorporating backbone flexibility into designs.

NMR spectroscopy has been employed for detailed analysis of the backbone dynamics of several mutants of *Streptococcal* protein Gβ1; the structural flexibility of these mutants is believed to significantly impact their respective stabilities compared to wild type [1]. Previously, we reported that a highly thermostable variant (Gβ1-c3b4) [2] dimerizes in solution; our 2.3 Å crystal structure shows that the interface is formed through a β-bridging interaction with antiparallel β2 strands. NMR relaxation experiments have revealed significant conformational exchange for all backbone amides in the β2 strand and the adjacent region of the helix (caused in part by the monomer-dimer equilibrium). In contrast, the backbone amides involved in both fast and intermediate timescale motions in the six-fold core mutant (FILIIW) are localized in residues close to mutations. Dynamics in both variants may be attributed to sidechain overpacking or the loss of specific interactions (such as aromatic ring stacking, which rigidifies the core of FILIIW). However, increased motion is likely due to new nonspecific interactions created by greater hydrophobic burial in the variants, which would lead to higher overall entropy (and therefore altered stabilities) compared to wild type. These data provide insight into the properties of protein sequences generated by ORBIT according to specific design criteria and highlight aspects of the algorithm that may require modification to produce variants with desired dynamic characteristics.

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265. Validating computationally designed *de novo* homodimers with X-ray crystallography

Possu Huang, Stephen L. Mayo

By combining our fast Fourier transform-based docking algorithm and our protein design algorithm, we have generated *de novo* homodimers by changing the surface properties of a previously redesigned homeodomain molecule. The formation of the homodimer is hydrophobicity-driven, and our current best dimer has a dissociation constant of ~20 μM, determined by analytical ultracentrifugation. We are in the process of determining the X-ray crystal structure of our latest version of the designed homodimers, as X-ray structural data is essential to validate the design. High quality crystals were obtained by vapor diffusion sitting drop experiments, and their X-ray data were collected at both home rotating anode sources and synchrotron sources. We were, however, unable to obtain a solution for the phases using molecular replacement methods due to the large number of molecules in the asymmetric unit. We are using a bromine-labeled variant to obtain experimental phases and are exploring other possible crystallization conditions for alternative space groups.

266. Designing calmodulin specificity: Incorporation of explicit negative design

Jennifer R. Keeffe[1], Stephen L. Mayo

Protein-protein interactions are essential to many processes within cells, including signaling pathways, the immune response and in the formation of the functional, oligomeric state of many proteins. The ability to design a protein with high affinity and specificity to a particular protein or peptide target would not only increase our understanding of protein-protein interactions, but also aid in the generation of new therapeutics, diagnostics and research tools.

Calmodulin (CaM) is an ideal system for studying the specificity of protein-protein interactions by computational design. CaM is a small $Ca2^{+}$-binding protein that binds to and regulates numerous proteins *in vivo* with high affinity, including smooth muscle myosin light chain kinase (smMLCK) and CaM kinase I (CaMKI). In addition, several high-resolution CaM-peptide complexes have been solved by X-ray crystallography.

Shifman and Mayo [1] successfully used the ORBIT computational design software to generate a CaM variant with increased specificity toward the smMLCK peptide. However, this feat was accomplished by optimizing the interface between CaM and the smMLCK peptide and did not use any negative design to disfavor the binding of other CaM-binding peptides. In addition, this variant did not discriminate between the smMLCK and CaMKI peptides [2], indicating that there is potential for improvement by including a negative design component against the CaMKI target.

Our current studies are focused on designing a CaM variant that can efficiently discriminate between the smMLCK peptide and the CaMKI peptide by incorporating explicit negative design. Toward this goal, we are using ORBIT to optimize 25 CaM positions in the peptide-binding interface. Using an optimization algorithm written by graduate student Benjamin Allen, the positions are simultaneously optimized on the CaM-smMLCK peptide structure (for a favorable solution) and the CaM-CaMKI peptide structure (for an unfavorable solution). We are currently investigating various negative design scoring functions and parameters to obtain the most favorable results. Future experimental validation of the computationally designed variants will include tryptophan fluorescence titrations to determine the binding affinity of CaM to the peptides.

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267. Computational design of a novel enzyme for catalysis of a pericyclic reaction

J. Kyle Lassila[1], Stephen L. Mayo

We are working towards the goal of fully automated enzyme design. In particular, we hope to apply computational protein design methods to the task of creating a completely novel catalyst for the Claisen rearrangement of chorismate to prephenate. Naturally catalyzed by the chorismate mutases, this reaction offers many desirable features as an early test of enzyme design methods. The reaction is a first-order sigmatropic rearrangement of a single substrate and has neither intermediate steps nor involvement of catalytic groups such as general acids or bases. The reaction has been extensively studied in many contexts - as a rare enzyme-catalyzed pericyclic process, as an essential step in the biosynthesis of aromatic compounds, and as a rare example of a reaction that occurs through identical mechanisms enzymatically and in solution. Our method of enzyme design involves identifying amino acid sequences likely to bind to an *ab initio* transition state structure of the chorismate-prephenate rearrangement. As a part of this process, we are testing new methods that allow translation and rotation of the transition state structure within a binding cavity while simultaneously optimizing protein side chain identity and conformation. When the design procedure was applied to a natural *Escherichia coli* chorismate mutase active site, a mutation was predicted that was shown experimentally to increase the catalytic efficiency of the enzyme [1]. These methods will be generally applicable to the design of ligand binding sites and enzyme active sites. The ability to computationally design new reaction catalysts can be expected to have important implications for organic synthesis, bioremediation, and biotechnology.

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268. Designing anti-viral proteins for preventive and therapeutic purposes

Matthew MacDougall, Alex L. Perryman*, Stephen L. Mayo*

The lectin cyanovirin-N (CV-N) from the cyanobacterium *Nostoc ellipsosporum* is a potent HIV-inactivating protein that binds carbohydrate moieties linked to the viral gp120 envelope protein. CV-N has proven effective in preventing rectal and vaginal HIV infections in macaques. CV-N also displays moderate activity against influenza and Ebola. At physiological temperatures CV-N is monomeric, but at room temperature it is a complex mixture of monomers and domain-swapped dimers, which complicates experimental analysis of this system. Despite considerable effort, no crystallographic data have been developed that provide insight into the mechanism by which the primary site of CV-N binds high mannose oligosaccharides on HIV envelope proteins. The Mayo Lab's ORBIT software was used to predict novel variants of CV-N that preferentially stabilize the monomeric structure. We will now synthesize and test these mutants. It is hoped that our variants will not display the crystal packing artifacts that prevent the acquisition of structural information on the mode by which a sugar binds to the primary site of CV-N. Further, we intend to investigate the effect of designed multimerization on the anti-viral activity of CV-N. These studies should at least facilitate a greater understanding of the structure-function relationships that govern the anti-viral function of this important protein. Results from these inquiries should help identify improvements to CV-N that will enhance its binding to HIV and therefore its utility as a therapeutic and preventive agent.

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269. Improved protein scoring functions using explicit solvent

Barry D. Olafson, Stephen L. Mayo

With our current design software, we can score the stabilities of various protein configurations very quickly and use this information to select proteins with desired properties. The scoring functions used with protein design algorithms rely on the use of rotamer libraries for amino acid sidechain placements and treat the effects of solvent implicitly, usually by evaluating surface areas of solvent-exposed atoms. At the end of the initial design process, we generate a number of viable 3D protein configurations (on the order of tens to hundreds) that are candidates for synthesis and experimental validation. We are now developing a follow-on procedure to reevaluate these final candidate configurations with more extensive scoring functions that treat the effects of the solvent explicitly and also relax the requirement that the sidechain positions conform to a given set of rotamers. The computational requirements for this new procedure are substantial, but the expectation is that by using a more rigorous scoring function we can improve the probability that we synthesize and validate the more useful configurations from the candidates generated by the design algorithms.

With the new methods, we explicitly add solvent molecules to completely envelope the candidate protein configurations. The scoring function estimates the relative stabilities of various protein configurations, which depends upon their internal structure and their additional stabilization or destabilization due to solvent interactions. Because we have explicit solvent atoms and their positions in the calculation, we are able to calculate the interaction between protein and solvent directly. In addition, we estimate the impact the presence of the protein has on causing the solvent water molecules to reorganize and add this contribution to the overall protein configuration score. It is essential that the combined system, protein and solvent, be allowed complete conformational freedom to allow the explicit water molecules to pack tightly to the protein surface, and for the amino acid sidechains to optimize their positions. Results to date indicate that these three general components (internal energies, protein interactions with solvent, and solvent reorganization) all contribute comparably for folded, stable proteins.

270. Bacteriorhodopsin-based bioelectronic devices: Developing optical data storage

Alex L. Perryman, Stephen L. Mayo

The Mayo group's approach to protein engineering has been used to design variants of bacteriorhodopsin (BR) that could be useful in three-dimensional optical memory devices. We are trying to create mutants of BR that display an increased yield of the O state of its photo-cycle, so that they can be used as the principal component in two-photon volumetric memory devices. Increasing the yield of the O state will increase the percentage of BR molecules that traverse the branched photo-cycle shunt to arrive at the Q state, which has a half-life of 5 to 20 years. This branched photo-cycle can be exploited for the generation of bioelectronic devices by having the ground state represent bit 0 and by utilizing both the P and the Q states for bit 1. If we are able to engineer variants of BR that display the desired properties, this could both increase the speed and decrease the size of computers by 300- to 1,000-fold, and it could also lead to the creation of the principal component that would make associative memory devices possible (i.e., it could facilitate the development of true artificial intelligence).

From the results of computational protein design calculations on the O-state crystal structure, the N-state crystal structure, and the ground-state crystal structure of BR, we proposed four mutants for synthesis and testing. Professor Bob Birge's lab at the University of Connecticut is now determining both the Q[O] (the yield of the O state) and the Qtotal of our proposed mutants. Qtotal is a relative measure of how well several different aspects of the photo-cycle have been improved, with respect to the

mutant's utility in the three-dimensional optical memory device.

271. Computational design of an enantioselective binding protein

Heidi K. Privett[1], Stephen L. Mayo

Fully automated enzyme design can be envisioned as the solution to many complex synthetic organic chemistry problems. Enzymes are ideal catalysts of organic reactions because of their extremely high rates of catalysis and their ability to perform a wide variety of chemical transformations with extreme regio- and enantioselectivity. With an increasing demand for enantiomerically pure, complex, biologically active compounds (i.e., drugs), synthetic chemists will have to look beyond standard chemical strategies in favor of the high yields and strict selectivity of enzymatic reactions. Designed enzymes will not be restricted to the reactions accessible by natural enzymes, broadening the range of possible substrates and products.

Kinetic resolution is a powerful method for transforming a racemic starting material into an enantiomerically pure product. Our goal is to show that an enzyme can be designed to carry out a kinetic resolution using the enantioselective hydrolysis of 2-phenyl-4-benzylphenyloxazolin-5-one (FOX) to produce *N*-benzoyl-L-phenylalanine as a model system. As a first step in the design of this enzyme, we will make a protein capable of selectively binding L-FOX and then design catalytic functionality into the optimized binding protein.

We have used the ORBIT protein design software to design an optimal binding pocket around an internally and externally flexible L-FOX structure using the hyperthermophilic *T. thermophilus* protein aspartate amino transferase (AspAT) as a scaffold. Within the context of a poly-alanine scaffold, the location and conformation of two important binding residues were first chosen based on the geometric constraints between the ligand and the sidechains that describe "optimal" contacts. The remainder of the binding pocket residues were repacked around the catalytic residues and the ligand, resulting in a twelve-fold mutant, named 1GCK-FFBP. We are currently evaluating the binding of FOX to 1GCK-FFBP using fluorescence anisotropy and attempting to determine the protein's effect on the enantiomeric enrichment of the product. Once binding of the ligand has been established, catalytic residues that support hydrolysis of the L-enantiomer of FOX can be introduced into the binding pocket.

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272. Preservation and diversity of function in designed combinatorial libraries of GFP

Thomas P. Treynor, Christina L. Vizcarra[1], Daniel Nedelcu[2], Stephen L. Mayo

Protein engineering is inefficient if each new protein functions identically to the one before it or not at all. In order to determine which of seven library-design algorithms best introduces new protein function without destroying it altogether, seven combinatorial libraries of green fluorescent protein (GFP) variants were designed and synthesized. Each was evaluated by distributions of emission intensity and color compiled from measurements made *in vivo*. Additional comparisons were made with a library constructed by error-prone PCR. Among the designed libraries, fluorescent function was preserved for the greatest fraction of samples in a library designed using a novel structure-based computational method. A trend was observed towards greater diversity of color in designed libraries that better preserved fluorescence. Contrary to trends observed for libraries constructed by error-prone PCR, preservation of function was observed to increase with a library's average mutation level among the four libraries designed with structure-based computational methods.

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273. Electronic structure and stability of fluorescent proteins from designed libraries

Thomas P. Treynor, Christina L. Vizcarra[1], Marco A. Mena[2], Daniel Nedelcu[3], Barry D. Olafson, Patrick S. Daugherty[2], Stephen L. Mayo

In order to investigate structure-function relationships with a reductive approach that is more thorough and nuanced than site-directed mutagenesis, one would ideally have many differently functional variants related by the permutation of a small set of mutations. In this way, one would isolate the effects of many individual mutations in many different contexts. Not just any combinatorial library will do; however, since random mutations at random positions are not likely to affect protein function except to destroy it altogether. By targeting positions close to green fluorescent protein's chromophore with mutations that have been computationally pre-screened for their effects on stability, we have generated considerable spectral diversity in a library of only 29 variants. We have sequenced many of these functional variants and show that three of nine designed mutations are chiefly responsible for much of the observed diversity of function. Using these mutations and the T203Y mutation that defines the class of yellow fluorescent proteins, we have constructed and characterized a quadruple mutant cycle for which substantial emission is observed *in vivo* for 12 of 16 variants. We find that the effects of these four mutations on emission and excitation spectra can be quite different depending on context. Many observations suggest that

vibronic mechanisms underlie much of the observed spectral changes.

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274. Improved continuum electrostatic and solvation for protein design

Christina Vizcarra[1], Frederick E. Tan[2], Stephen L. Mayo

Computational protein design protocols use energy functions to score interactions between pairs of sidechains in the context of a given fold. These energy functions guide the search for a sequence or library of sequences that will stabilize the folded structure. The accuracy of the energy function is a limiting factor in designing stable, well-folded proteins and can also hinder efforts in enzyme design. We are interested in improving the energy terms that correspond to electrostatic interactions and the solvation of polar groups in the ORBIT protein design program. To this end, we have incorporated into ORBIT the Finite Difference Poisson Boltzmann (FDPB) model, which is generally considered a standard for accuracy among continuum electrostatics models. In order to use the FDPB model for protein design, we formulated it to be compatible with 'two-body' scoring functions, where each 'body' is a sidechain conformation. We have found that, for the FDPB solver DelPhi, using approximate representations of the protein surface and a two-body energy model, it is possible to reproduce the results of traditional FDPB calculations in which the entire dielectric environment around the protein is defined. Using this model, we have designed a variant of the all alpha-helical protein, *Drosophila engrailed* homeodomain, by only varying the identities of amino acids on the protein surface. Experimental characterization of this designed sequence has shown that it is thermophilic and unfolds at a higher temperature than sequences designed using electrostatic heuristics to bias sequence composition. This result shows that the two-body requirement of most protein design protocols does not preclude use of an FDPB model. This study also underscores the power of electrostatic optimization of a protein's surface as a means to stabilize the folded state.

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275. Force field development for protein design

Eric Zollars[1], Stephen L. Mayo

Protein design is an exceptionally difficult problem characterized by unique complications. Necessary restrictions such as a fixed backbone and discrete side-chain conformations require different considerations of structure-energy relationships than other fields of protein simulation. This structure-energy relationship is the focus of our recent research, which hopes to address issues including the identity of the forces that lead to protein stability and the relative strengths of these forces. Additionally, the mathematical representation of these forces must be relatively simple to allow for tractable design calculations.

It is thought that local side-chain-backbone interactions are responsible for a great deal of the basic structure of proteins. A purely physical representation of these interactions is complex due to the small distances involved. An accurate statistical treatment of these interactions may allow for better designs in areas of protein secondary structure. We are therefore investigating the utility of including a statistically-based secondary structure propensity term in the force field.

An entropic term has also been added to the forces considered during a design calculation. It is often observed that an enthalpic-entropic "balance" occurs in proteins. Simply, a protein's overall stability is a result of contributions from entropic and enthalpic components. Since most of the other energy terms used in protein design are enthalpic in nature, the addition of an entropic term is a useful feature.

A previous component of this research included the development of a method to allow for quick parameterization of the energy terms used in the design calculations. The use of a large database of thermodynamic data to arrive at useful parameters was dropped because of the large relative errors within the database. The current method is based on the assumption that naturally occurring protein sequences are nearly optimal for their respective structures [1]. This method will achieve parameters that are not perfectly optimized, but because of the conservative nature of this approach, the parameters are expected to lead to stable designed proteins.

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Summary: Our laboratory is interested in the origin and evolution of the biochemical functions that characterize modern life on Earth, and we seek to develop ways of probing the coevolution of metabolism with Earth's near-surface environments. Understanding how modern microorganisms with archaic metabolisms function is a necessary step toward this end. Due to the basic nature of our questions, and the fact that many microenvironments on Earth today are anaerobic (such as the environments that characterize the majority of bacterial infections), this path of inquiry leads inexorably to insights about cellular electron transfer mechanisms that have broad relevance.

Because rocks provide the primary record of ancient events and processes, microbe-mineral interactions are our focus. In particular, we investigate how bacteria catalyze mineral formation, transformation, and dissolution, with a focus on how these processes are related to cellular energy generation and how they affect the geochemistry of their environment. For every metabolism that we study, we work with model organisms that we can genetically manipulate. Through a combination of classical genetic, biochemical, and molecular biological approaches, we identify the genes and gene products that control the processes of interest. By understanding the way extant organisms function at the molecular level, we hope eventually to gain insights into the evolution of ancient metabolic and biomineralization pathways, interpret the chemical signatures of early life found in the geologic record, and quantitate/control the contribution of microbes to present-day geochemical cycles.

276. Spatiometabolic stratification of *Shewanella oneidensis* biofilms

T.K. Teal, D.P. Lies, B.J. Wold, D.K. Newman

Bacteria are typically viewed as living a primarily planktonic lifestyle. In the last decade, however, it has become more apparent that bacteria spend much of their lives as surface-attached microbial communities, i.e., biofilms, and it has been suggested that this biofilm lifestyle may account for the remarkable persistence of bacterial populations in the face of changing environmental conditions. Biofilms are composed of many hundreds of cells, each of which experiences it own microenvironment due to strong chemical gradients that are established by metabolism and diffusion. Biofilm communities are therefore heterogeneous and spatially stratified, such that the environments within different regions of the biofilm can vary markedly. Although much is known about how biofilms form, develop and detach, very little is understood about how these events are related to metabolism and its dynamics. It is commonly thought that large sub-populations of cells within biofilms are dead, but it may be that within the growth inactive domains of biofilms, significant populations of living cells continue to persist and retain the capacity to dynamically regulate their metabolism.

To explore the spatiometabolic stratification of developing and mature biofilms, we selected *Shewanella oneidensis* strain MR-1, a biofilm-forming, facultative anaerobe with remarkable metabolic versatility. *S. oneidensis* can use oxygen and many other lower potential substrates, including metals, as electron acceptors in respiration, making it an attractive experimental system in which to explore domains of metabolism within a biofilm. To study this metabolic patterning, we have fused different promoters to the unstable green fluorescent protein *gfp(AAV)*, such that the activity of the promoter can be tracked by measuring fluorescence levels *in vivo* over the course of biofilm development. The ribosomal promoter *rrnB* P1 is being used to measure growth activity levels. Fluorescence levels from this promoter/GFP system have been shown to be a good indicator of cell growth and replication. To determine if metabolism (i.e., protein synthesis and energy generation) might occur in regions where cells are not actively growing, the promoter for *mtrB*, a gene expressed under anaerobic conditions, is used. We are growing the biofilms in a flow cell system and imaging them using confocal fluorescence microscopy. Using this technique we can correlate the level of fluorescence we see using microscopy with metabolic activity and can determine the regions of the biofilm in which cells are the most metabolically active as well as explore how quickly this state can change in response to environmental perturbations. We have developed a quantitative routine to compare these domains of activity among independently grown biofilms.

We have found that *S. oneidensis* biofilm structures stereotypically stratify with respect to growth activity and metabolism as a function of their size. Within domains of growth inactive cells, genes typically

upregulated under anaerobic conditions are expressed well after growth has ceased. These findings reveal that, far from being dead, the majority of cells in mature *S. oneidensis* biofilms have actively turned on metabolic programs appropriate to their local microenvironment and developmental stage. We are continuing to investigate what defines and controls the capabilities and activities of this growth state, as this information is not only essential for understanding basic aspects of biofilm biology, but is also is also extremely relevant for applications that aim to exploit the metabolic activity of biofilms for energy conversion and other purposes

277. Characterization of a bacterial arsenate respiratory reductase

Davin Malasarn, Dianne K. Newman

The arsenate respiratory reductase from the gram-negative bacterium *Shewanella* sp. strain ANA-3 represents a highly conserved group of enzymes that allow some microbes to gain energy by reducing arsenate, As(V), to arsenite, As(III), in the absence of oxygen. This process is environmentally relevant (1), and understanding its biochemical mechanism may provide valuable information about how anaerobic respiration evolved to allow the utilization of such diverse substrates as arsenate, nitrate, DMSO, and selenate.

We have overexpressed the arsenate respiratory reductase from ANA-3 in *E. coli* and isolated wild-type and mutant forms for characterization. The reductase is stable as a heterodimer consisting of ArrA and ArrB, and both subunits are required to transfer electrons from the electron donor methyl viologen to arsenate. Alternate substrates such as selenate, nitrate, sulfate, tungstate, molybdate, and phosphate do not get reduced by the enzyme, nor do they serve to inhibit arsenate reduction. ArrA contains a twin-arginine translocation (TAT) signal, suggesting that it is secreted to the periplasm of ANA-3 (2). However, this signal is not required for proper protein synthesis, and a truncation lacking the TAT signal is still active. In contrast, W293A and K295A mutations result in complete loss of activity. The role of W_{293} and K_{295} is unknown, and current efforts are focused on the crystallization of ArrAB in the hopes of identifying the substrate-binding site of the reductase. Additionally, kinetic, analytical, and spectroscopic characterization is being completed, and systematic mutational analysis is being performed to identify additional residues that are important for function. Lastly, as a member of the DMSO reductase family, ArrAB requires molybdenum, iron, and sulfur to function. Through our studies, we hope to understand the coordination and redox properties of these cofactors. This will allow us to understand how enzymes within this family are individually fine-tuned for each mode of respiration.

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278. Physiology and mechanisms of phenazine redox cycling in *Pseudomonas aeruginosa*

Alexa Price Whelan, Dianne K. Newman

Several members of the genus *Pseudomonas* excrete of a class of brilliantly colored compounds, called the phenazine derivatives, in stationary phase. These redox-active compounds are taken up and reduced by the bacteria, and then shuttle to extracellular substrates for reoxidation. Given that electron transfer reactions fuel cellular metabolism, and stationary-phase cells are often thought to be limited for metabolic substrates (such as the terminal electron acceptor oxygen), it is often thought that intracellular reductants accumulate in stationary phase due to the lack of a suitable electron acceptor. We hypothesized that phenazine reduction could allow for disposal of excess reducing power, and significantly affect the intracellular redox state of stationary-phase cells. To test this, we measured the redox state of NAD(H), a major intracellular redox cofactor, throughout the exponential and stationary phases of growth for the phenazine-producing opportunistic pathogen *Pseudomonas aeruginosa* PA14, as well as a *P. aeruginosa* mutant that is unable to produce phenazines. We found that the NAD(H) pool was significantly more reduced in stationary phase in a phenazine-null mutant relative to the wild type. This result implies that *P. aeruginosa* is able to utilize phenazine reduction as a mechanism for the maintenance of redox homeostasis. Consistent with this, addition of pyocyanin, a major phenazine produced by *P. aeruginosa*, to the phenazine-null mutant leads to a decrease in the NADH/NAD+ ratio.

We are interested in elucidating the molecular mechanisms underlying phenazine redox cycling in *P. aeruginosa*. We plan to perform a mutant screen to identify genes required for phenazine reduction. Additionally, we have identified genes in the sequenced *P. aeruginosa* genome that potentially encode enzymes responsible for the reduction of flavins, another class of common intracellular redox cofactors. Flavins bear strong structural and chemical similarities to phenazines, leading us to postulate that enzymes annotated as putative flavin-reducing proteins may actually serve to catalyze phenazine reduction. We will evaluate this hypothesis through targeted mutagenesis and characterization of whole-cell and cell-free extract phenazine reduction activity.

279. Production and function of 2-methylhopanoids in *Rhodopseudomonas palustris*

Sky Rashby, Alex Sessions, Dianne K. Newman*

The diagenetic derivatives of 2-methylbacteriohopanepolyols have been utilized extensively as biomarkers for cyanobacteria and, by association, oxygenic photosynthesis. Recently, we have identified quantitatively significant production of these compounds in the alpha proteobacterium *Rhodopseudomonas palustris* growing phototrophically

under anoxic conditions. This suggests that these compounds cannot be used as markers of oxygenic photosynthesis, and cause us to rethink when this metabolism evolved. Additionally, preliminary data suggest that specific modes of phototrophic growth may promote the production of these compounds in *R. palustris*, implying physiological functions that may refine our use of bacteriohopanoid biomarkers as paleoenvironmental proxies. Our future goals are to identify their cellular function, as well as to understand their biosynthetic pathway.

[*]*Assistant Professor of Geobiology, Caltech*

280. Studies of stromatolite morphogenesis using *Synechocystis* PCC6803

Mike Tice, John Grotzinger[*], Dianne K. Newman

Stromatolites are laminated, three-dimensional structures found in sedimentary rocks deposited as early as 3.5 billion years ago. Many macroscopic morphologies are known, including domes, cones, and branched or unbranched columns, and sizes range from millimeters to tens of meters. Nearly all known modern analogs are dominated by microbial mats, and consequently, nearly all geologic interpretation of these structures has been in terms of benthic microbial communities thought to dominate construction and morphogenesis. However, very little is known about how specific biological processes contribute to ultimate stromatolite shape, or why stromatolites should develop three-dimensional relief at all. Indeed, modeling suggests that many types of stromatolites could be formed by simple microscopic growth laws that may not be unique to specific groups of organisms. We are conducting experiments designed to explore macroscopic spatial arrangement of populations of several species of bacteria under a range of autotrophic and heterotrophic growth conditions. Preliminary results suggest that a diffusible signal may be involved in organizing colonies of a unicellular cyanobacterium, *Synechocystis* PCC6803. Future experiments will examine the effect of chemical precipitation simultaneous to mat growth on overall mat/stromatolite morphology.

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281. Role of secondary metabolites in biofilm communities

Itzel Ramos, Dianne K. Newman

Secondary metabolites are often defined as chemical compounds that are non-essential for growth, development or survival of the producing organisms. This idea has relegated their function(s) to a merely adaptive advantage conferred by their known anti-microbial activities. Recent studies, however, have uncovered the possibility that secondary metabolites have primary physiological roles. Phenazines, for instance, which are redox-active compounds produced by pseudomonad species, seem to act as signaling factors up-regulating a number of different genes (1). Also, because of their redox properties, it has been suggested that phenazines might act as electron shuttles to power oxidation of NADH when oxygen is limited (2). We are presently investigating the physiological functions of phenazines in biofilm communities of *Pseudomonas aeruginosa*. Biofilms are thought to be the most common form of bacterial growth in nature. In these structures oxygen limitation could be a challenge for bacterial survival, but phenazines might be participating actively to maintain redox homeostasis. First, we are analyzing biofilm development of mutant strains partially or totally impaired in their ability to produce phenazines. Second, to track expression of genes involved in phenazine production, we are constructing transcriptional fusions of unstable florescent proteins. This will allow us to determine where and when phenazines are produced during biofilm development, as well as to investigate the effect of different environmental factors in the production of phenazines. Also, taking advantage of the fluorescent properties of these compounds, we are exploring the possibility to detect phenazines directly in biofilms using two-photon microscopy. Our general hypothesis is that during the course or biofilm development and maturation, the production of phenazines will follow specific patterns depending on the local environmental conditions, specifically oxygen concentration. These studies will bring insight into how phenazines might contribute to survival of biofilm communities.

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282. Extracellular electron transfer by bacteria

Doug Lies, Dianne K. Newman

Certain bacteria are known to frequently transfer electrons outside their cell to insoluble or inaccessible electron acceptors using either direct surface-mediated electron transfer or soluble redox-active electron shuttles. We have been studying this process in two model systems: surface-mediated reduction of dimethyl sulfoxide (DMSO) by *Shewanella oneidensis* MR-1 and production of the soluble redox-active phenazine compound pyocyanin by *Pseudomonas aeruginosa* PA14 biofilms.

In the *S. oneidensis* system, during the characterization of the extracellular nature of the DMSO reductase complex, we determined that, although localized on the exterior surface of the cell, the complex was protease resistant in whole cells or cell lysates, unlike the case for the highly related DMSO reductase from *E. coli*. Additionally, we developed quantitative assays for DMSO reductase activity and analyzed *S. oneidensis* mutant strains defective in components co-expressed with the DMSO reductase, as well as mutants defective in protein export that affect the DMSO reductase of this organism.

In the *P. aeruginosa* system, we are using a novel flow cell capable of performing electrochemical analyses (the E-Tongue III) to analyze and electrochemically manipulate biofilms of this organism. Our initial

experiments have determined when pyocyanin production by strain PA14 is electrochemically detectable in the E-Tongue biofilms and we are currently correlating this analysis with detection of phenazine biosynthetic gene expression in the biofilms using fluorescent transcriptional reporters. Additionally, we are examining the metabolic state of PA14 cells in biofilms using a ribosomal RNA fluorescent transcriptional reporter and whether we can alter that state by electrochemical water-splitting generation of H_2 or O_2 gas beneath the biofilm with the E-Tongue. Follow-up studies will examine whether pyocyanin production by PA14 influences the metabolic state under these conditions.

283. *Pseudomonas aeruginosa*'s SoxR is activated by phenazine antibiotics and controls genes that regulate phenazine shuttling

Lars E.P. Dietrich, Alexa Price Whelan, Dianne K. Newman

The transcription factor SoxR is well characterized in *E. coli* as a stress response regulator. Upon exposure to superoxide-generating compounds, SoxR induces the transcription of more than 60 genes involved in protection from harmful oxygen radicals. Homologues of *E. coli* SoxR are present in a variety of Gram-negative and Gram-positive bacteria, suggesting a conserved response to superoxide. The opportunistic pathogen *Pseudomonas aeruginosa* apparently does not comply with this paradigm: *P. aeruginosa*'s SoxR does not control any of the genes typically involved in the conversion of superoxide, but instead regulates the expression of two transporters and a monooxygenase. This provoked two questions: (i) What is the function of the SoxR regulon in *P. aeruginosa*? (ii) Does SoxR respond to signals other than superoxide? A defining characteristic of *P. aeruginosa* is the production of phenazines. These are colorful, redox-active antibiotics that shuttle in and out of the cell. Reduced phenazines can generate superoxide, which contributes to virulence in eukaryotic infection models. Using Affymetrix GeneChips we analyzed the effect of the phenazine pyocyanin on *P. aeruginosa* gene expression and found a specific response with only 22 genes being up regulated, amongst them the complete SoxR regulon. This established pyocyanin as an endogenous activator of SoxR. To address the function of its regulon we deleted *soxR* and detected a dramatic defect in the cell's ability to reduce phenazines. This could be due to the lack of SoxR itself or to the missing upregulation of SoxR target genes. The latter turned out to be correct as deletion of one of the target genes, encoding a MFS (major facilitator superfamily) transporter, also prevented phenazine reduction. We speculate that the MFS transporter is required for phenazine import. Based on our findings that phenazines activate SoxR and that the SoxR response is required for the reduction of phenazines, we propose that in *P. aeruginosa* SoxR and its regulon adapted to specifically ensure the redox shuttling of phenazines.

284. A role for phenazine antibiotics in contributing to iron acquisition

Yun Wang, Dianne K. Newman

In aerobic environments, Fe is present as insoluble Fe(III) minerals, causing the available Fe concentration ($[Fe^{3+}(aq)] = 10^{-17}$ M) to be ten orders of magnitude lower than the concentration needed for bacterial growth ($>10^{-7}$ M). It is well known that *P. aeruginosa* can acquire iron via the production of siderophores (pyoverdin and pyochelin) to dissolve Fe(III) minerals by forming Fe(III)-siderophore complexes in solution. We are testing the hypothesis that *P. aeruginosa* may be even more effective at acquiring iron via the production of redox-active phenazine antibiotics, which can function as extracellular electron shuttles to reduce and subsequently dissolve Fe(III) minerals. Our studies suggest that the redox potentials of phenazines produced by clinical- and plant-associated *Pseudomonas* spp. are sufficiently low under environmental conditions to promote Fe(III) mineral reduction. Phenazine reactivity is very well correlated to its thermodynamic properties, more specifically, the phenazine structure with lower reduction potential yields higher rates of Fe(III) reduction. Phenazine reactivity is influenced by environmental factors, *e.g.*, the reaction rate is higher at lower pH; the reaction is more likely to happen in the presence of the poorly crystalline Fe(III) (hydr)oxide than the highly crystalline hematite. Phenazines and siderophores appear to promote Fe(III) dissolution synergistically, and we are working on understanding the mechanism responsible for this phenomenon.

285. Genetic identification of phototrophic Fe(II) oxidation systems

Yongqin Jiao, Laura Croal[*]*, Dianne K. Newman*

The Fe(II) oxidation system in *Rhodopseudomonas palustris* TIE-1. Phototrophic Fe(II) oxidizing bacteria couple the oxidation of ferrous iron [Fe(II)] to reductive CO_2 fixation using light energy, but little is known about the molecular basis for this process. Using *Rhodopseudomonas palustris* strain TIE-1 as a model organism, we report the discovery of three genes necessary for phototrophic Fe(II) oxidation. *De novo* synthesis of a unique soluble *c*-type cytochrome of approximately 40 KDa was identified under Fe(II) grown conditions. Mass spectrometry indicates that it is a decaheme *c*-type cytochrome present in the sequenced stain *R. palustris* CGA009. Using primers based on CGA009, three consecutive open reading frames (ORFs), designated as *pio* genes (phototrophic iron oxidation), were found in TIE-1. The first ORF, named *pioA*, encodes the *c*-type cytochrome over-expressed under Fe(II) grown conditions. *pioA* contains a signal sequence characteristic of secreted proteins. The amino acid sequence of PioA exhibits 38% identity to MtrA in *Shewanella oneidensis* MR-1. Two ORFs were found directly downstream of *pioA*, and were designated *pioB* and *pioC*. RT-PCR results indicate that these three ORFs are arranged in an operon. *pioB* encodes a putative outer membrane beta-barrel

protein and the N-terminal sequence contains a potential signal for secretion. PioB is 21% identical to the outer membrane protein MtrB in *S. oneidensis* MR-1. *pioC* encodes a putative high potential iron sulfur protein (HiPIP) and it contains a twin-arginine translocation (tat) signal. The amino sequence of PioC exhibits 44% identity to a putative iron oxidase (Iro) from *Acidothiobacillus ferroxidans*. Deletion of *pioABC* resulted in the loss of Fe(II) oxidation activity but did not affect growth on other substrates. Complementation studies confirmed that this phenotype was due to the loss of the *pio* operon. Deletion of *pioA* alone resulted in loss of almost all Fe(II) oxidation activity, however, deletion of either *pioB* or *pioC* alone resulted in partial loss of Fe(II) oxidation activity. These results suggest that proteins encoded by the *pio* operon interact, and are essential and specific for phototrophic Fe(II) oxidation in *R. palustris*.

The Fe(II) oxidation system in *Rhodobacter* sp. SW2. In collaboration with Laura Croal, we investigated the Fe(II) oxidation system in another purple nonsulfur bacterium *Rhodobacter* sp. SW2 that had previously been isolated by Fritz Widdel *et al*. For practical purposes, SW2 is not amenable to traditional genetic analysis because it does not form single colonies on agar plates. Thus, to identify genes involved in Fe(II) phototrophy by SW2, we expressed a genomic cosmid library of this strain in its close relative *Rhodobacter capsulatus* SB1003 and screened cell suspensions of each clone for enhanced, light-dependent, Fe(II) oxidation activity. We identified a three-gene operon, *foxEYZ,* that confers enhanced Fe(II) oxidation activity when heterologously expressed in SB1003. The first gene in this operon, *foxE*, encodes a *c*-type cytochrome with no similarity to other known proteins. Expression of *foxE* alone confers light-dependent Fe(II) oxidation activity to SB1003, however, maximal activity is achieved when *foxE* is expressed with the downstream genes *foxY and foxZ*. Sequence analysis suggests that *foxY* encodes a protein containing the redox cofactor pyrroloquinoline quinone. The third gene in this operon, *foxZ*, is predicted to encode a protein with transport function. Together, our results suggest that the *foxEYZ* operon encodes proteins that play key roles in phototrophic Fe(II) oxidation in *Rhodobacter* sp. SW2.

Environmental/geological relevance of phototrophic Fe(II) oxidation in the deposition of banded iron formations (BIFs). Both molecular hydrogen (H_2) and Fe(II) can serve as electron donors for anoxygenic photosynthesis and are predicted to have been present in the atmosphere and ocean of the Archean in quantities sufficient for phototrophic energy metabolism. If H_2, given its more favorable redox potential compared to Fe(II), were the preferred substrate for anoxygenic phototrophs, the presence of H_2 in an Archean atmosphere/ocean may have precluded the involvement of phototrophs capable of Fe(II) photoautotrophy in the deposition of BIFs. In collaboration with Laura Croal, we investigated the effect of H_2 on rates of Fe(II) oxidation by cell suspensions of *R. palustris* TIE-1 and *Rhodobacter* sp. SW2. We found that Fe(II) oxidation still proceeds under an atmosphere containing ~3 times the maximum predicted concentration of H_2 in the Archean, as long as CO_2 is abundant. Thus, it seems that the presence of H_2 in an Archean atmosphere would not have posed a barrier to Fe(II) photoautotrophy, given the high estimates of CO_2 at this time, consistent with the hypothesis that Fe(II) oxidizing phototorphs may have catalyzed ancient BIF deposition.

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286. Purification and spectrophotometric studies of a putative Fe(II) oxidoreductase in *Rhodopseudomonas palustris TIE-1*

Christine Romano, Dianne K. Newman

Oxidation of ferrous iron [Fe(II)] by anoxygenic photoautotrophic bacteria is thought to represent one of the most ancient forms of metabolism. The electron transport pathway for phototrophic Fe(II) oxidation, however, is not well understood. Previously, we discovered a three-gene operon (*pioABC*) necessary for phototrophic Fe(II)-oxidation in an Fe(II)-oxidizing bacterium *Rhodopseudomonas palustris* TIE- 1. Based on these results, we hypothesize that *pioA*, encoding a multi-heme *c*-type cytochrome, is the Fe(II) oxidoreductase that transfers electrons from Fe(II) to subsequent electron carriers during TIE-1 growth on Fe(II). Currently, I am trying to overexpress and purify PioA in *E. coli* so that the mechanism by which it oxidizes Fe(II) can be unraveled. *pioA* was PCR amplified and cloned into the overexpression vector pETGEX. The resulting plasmid was sequenced and will be transformed into *E. coli* BL21 for overexpression and purification.

In order to assist the purification of PioA, I am also developing an activity assay for Fe(II) oxidation by TIE-1 cell extracts. The spectrum of the soluble fraction isolated from TIE-*1* lysate contained peaks indicative of *c*-type cytochromes. These *c*-type cytochromes were first oxidized by treatment with potassium ferricyanide. After dialysis to remove excess potassium ferricyanide, samples will be reacted with Fe(II) under anaerobic conditions and changes in cytochrome *c* peaks (from the oxidized form to the reduced form) will be monitored spectrophotometrically to determine whether some *c*-type cytochrome(s) in the soluble fraction are able to react with Fe(II). As a negative control, these experiments will also be performed on the extracts of a *pioA* deletion mutant of TIE-1.

287. Anoxygenic phototrophic Fe(II) oxidation as a mechanism for carbon acquisition

Nicky Caiazza, Dianne K. Newman

Rhodobacter capsulatus SB1003 is a purple non-sulfur, α-proteobacteria that can oxidize soluble Fe(II) in a light-dependent manner. Interestingly, *R. capsulatus* SB1003, unlike its close relative *Rhodobacter ferrooxidans* SW2, cannot grow photoautotrophically using soluble Fe(II) as a sole source of electrons. However, it can grow photoautotrophically using Fe(II) as a source of electrons if the Fe(II) is chelated with an agent such as nitrilotriacetic acid (NTA). It is feasible that the chelator facilitates Fe(II) oxidation by altering the reduction potential of the Fe(II)/Fe(III) redox couple and/or by preventing cell encrustation with Fe(III) minerals upon biological Fe(II) oxidation.

We have tested biological chelators to see if they can stimulate Fe(II) based photoautotrophic growth in *R. capsulatus* SB1003. When Fe(II)-citrate is provided as an electron source it resulted in high levels of bacterial growth. While citrate is an excellent nutrient source for many microbes it is not used by *R. capsulatus* SB1003 as a sole source of carbon and energy under phototrophic conditions. However, Fe(III)-citrate is a photochemically active species and in the presence of light is rapidly converted to Fe(II) and acetoacetic acid. We have begun to elucidate a mechanism whereby *R. capsulatus* SB1003 is able to acquire energy from citrate, an environmentally relevant and abundant compound that was once thought to be inaccessible to this organism. We propose that bacterial oxidation of Fe(II)-citrate leads to the formation of Fe(III)-citrate and that the later is photochemically oxidized leading to the formation Fe(II) and acetoacetic acid. Acetoacetic acid can be used by *R. capsulatus* SB1003 as a substrate for growth.

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Summary: We are interested in two groups of animal viruses, the alphaviruses and the flaviviruses. These viruses contain an RNA genome of about 11,000 nucleotides and are enveloped, having a lipid envelope that surrounds an icosahedral nucleocapsid. Both alphaviruses and flaviviruses are vectored by mosquitoes or other hematophagous arthropods and infect both their arthropod vectors as well as a wide range of vertebrates. We wish to understand the replication of alphaviruses and flaviviruses at a molecular level and to study the evolution of these two virus groups and more broadly the evolution of all RNA viruses.

The flaviviruses contain about 70 members, most of which are important human pathogens causing hundreds of millions of cases of human disease annually, which include hemorrhagic fevers and encephalitis. West Nile virus, which first appeared in the Americas seven years ago in New York state and then spread rapidly over most of the United States, is a member of this family. Other important flaviviruses include yellow fever virus and dengue virus. The alphaviruses consist of about 30 members of which several cause encephalitis, polyarthritis, or febrile illnesses in humans.

Although the alphaviruses and flaviviruses are not closely related, the structures of the virions in the two groups have been found recently to be related. One of the two glycoproteins present on the exterior of the virions, called E in the case of flaviviruses and E1 in alphaviruses, are structurally identical. There are other similarities in the structures of the virions in the two groups, including the formation of a heterodimer between E or E1 and the second external glycoprotein that function in an equivalent fashions. It seems clear that the virus structures, although they differ in important ways, are derived from a common ancestral source. We have suggested that this common structure may be important in the life of these viruses as arboviruses, capable of infecting both hematophagous arthropods and vertebrates.

We have started an ambitious collaboration with structural biologists at Purdue University to determine the structures of alphaviruses and flaviviruses to high resolution, and to determine the structures of all of the proteins encoded by the viruses to atomic resolution. We have now determined the structure of dengue virus to 9.5 Å and the structure of immature dengue virus that contains uncleaved prM to 11 Å. This resolution is high enough that the glycoproteins can be positioned with confidence in the virus particle, that carbohydrate side chains of the glycoproteins can be seen, and even the membrane spanning domains that anchor the glycoproteins can be identified and traced. One interesting result from such analysis is that protein E has a different bend in the virion from that in the immature virus, and that this bend also differs in the crystallized forms of E. Of even greater interest is the fact that E undergoes large structural rearrangements upon conversion of the immature virus to the mature form and again upon conversion of the mature virus to the fusion-competent form. We are extending these structural studies by constructing viruses with mutations in various proteins in order to study the interactions among proteins required for virus assembly. Such studies are important not only for our understanding of virus replication, but will be useful in the design of antivirals or of vaccines to control these important disease agents.

288. The function of yellow fever envelope protein in virus assembly

Marlene Biller, Brian Blood, Maria Farkas

Yellow fever belongs to the *Flaviviridae* family, which are enveloped positive-stranded RNA viruses. The viral envelope (E) protein of yellow fever virus has several major functions, including receptor attachment and membrane fusion, and it also carries the major antigenic epitopes leading to a protective immune response. E proteins in mature virions form 90 head-to-tail dimers that lie parallel and completely cover the viral membrane. Dimers of E protein have also been fitted into the cryoelectron density envelope of Dengue virus and there has been interest in determining how important dimer formation by E protein is for the overall configuration (and perhaps the stability) of flaviviruses. The E protein has an elongated shape and is composed of three major parts: a central domain that contains the N-terminal region (domain I), the fusion and dimerization domain (domain II), and a C-terminal putative receptor-binding domain (domain III). A set of mutants have been made to investigate dimer formation. These mutants are in contact regions of the homodimer and were carefully selected, based on where they are located in the protein and whether they are conserved among flaviviruses.

We have nine mutations in the E protein to the current time. Three are in domain II: F1 mutation E251 K, F2 mutation E251Q, and F3 mutation S253P to insert a helix breaker. There are four in domain III: F4 deletion aa 313-314, F5 Deletion aa. 313-316, F7 Mutation T311A, F8 Mutation V319D. F6 consists of an insertion of three Ala residues at aa 155 in domain I. The mutations were made in a transient expression vector, and all of them have been introduced into the full-length yellow fever virus clone (pACNR vector). RNA is transcribed *in vitro* and used to transfect BHK cells. RNA replication is detected by

immunofluorescence, and virus infectivity is checked by plaque assay.

All the mutants in domain III altered the infectivity. F4 and F5 were not infectious. A mutant called F4a was made, where aa T313-G314 were replaced by alanines, instead of deleted, as in F4. F4a had very low infectivity. Immunofluorescence showed primarily single cells transfected, so the infectivity is probably caused by revertants. RT-PCR will be done to check the sequence of the plaque. It is interesting that this change in F4a (TG to AA) affected the virus infectivity this much. F7 and F8 also had low infectivity. The infectivity of F8 was probably caused by revertants. The domain III area is very important for the virus, and the areas we have been looking at probably cover the fusion peptide in the opposite E protein. Mutant F1 in domain II had nearly no infectivity when titered with plaque assay of 1h transfected cells, but become more infectious when doing a plaque assay of 3-day transfected cells, probably due to revertants. Mutants F2 and F3 in domain II had low infectivity. Mutant F6 in domain I was less infectious than wild type and gave plaques of significant smaller size. Next we will purify the virus and crosslink the E proteins to see if they produce dimers. We have also started to do some double mutants. The ones we are most interested in at the moment are F1, F4a and F8. We are going to put F4a together with F1 and with F8, to see if that affects the dimerization. Since the mutants we are most interested in are the ones with no or very low infectivity, the challenge for us now is to produce enough particles of those mutants to send to Purdue for cryo-electron microscopic reconstruction.

289. Generation of alphaviruses containing uncleaved PE2 glycoproteins

Marlene Biller, Brian Blood, Pritsana Chomchan

We are currently working with three different alphaviruses: Sindbis, Ross River and Aura virus. They are members of the *Togaviridae* family. They contain a plus-strand RNA genome in an icosahedral nucleocapsid, surrounded by a lipid envelope. Alphaviruses contain 240 copies each of three structural proteins: two transmembranal glycoproteins (E1 and E2) and a capsid protein. The structural proteins of Alphavirusea are translated from a 26S subgenomic RNA as a polyprotein. The capsid protein is cleaved by the viral capsid autoprotease shortly after synthesis, and in ER PE2 (precursor of E2) and E1 are produced, and rapidly associates to (PE2/E1)3 heterotrimers. PE2 is cleaved in the post Golgi compartment, before the trimers are delivered to the plasma membrane, by furin, to form E2 and E3. The last four amino acids of E3 (aa 61-64 in SIN and RR) contain the furin recognition sequence (R-X-R-R or R-X-K-R) in almost all alphaviruses. PE2 containing particles have been produced for Semliki Forest virus and shown to be non-infectious.

We want to produce PE2 containing particles of Sindbis, Ross River and Aura for structural studies. We are removing the furin cleavage sites between E3 and E2 in full-length cDNA clones. For Sindbis we have created four mutants: SBI1 mutate R64S, SBI2 R64L, SBI3 (creates a N-glycosylation site) andSBI4 (deletion of SKR62-64). For Ross River we have done two mutants RRI2 (creates a N-glycosylation site) and RRI3 (deletion mutant). For Aura virus we plans to do the N-glycosylation mutant and the deletion mutant. We have cloned all the Sindbis and Ross River mutants into full-length cDNA clones.

Sindbis results: They were infectious, but grew to a lower titer than wt. We have determined that the virus produced did contain PE2 by protein gel electrophoresis and with Western blots stained with anti E2 antibody. We have sent preparations of SB I3 and SBI4 purified on sucrose gradients to Purdue University for EM and Cryo EM and are awaiting the results. We are very interested in the N-glycosylation mutants, because the additional glycan might be visible on the Cryo-EM, and thereby showing the furin cleavage site is in the particle.

Ross River result: The Ross River deletion mutant was not infectious. We have collected and purified particles produced from electroporation. The N-glycosylation mutant was less infectious that wt. We have done infections and purified the virus. These samples are also sent to Purdue University for EM and Cryo EM.

290. Flexibility of yellow fever E protein

Marlene Biller, Ashley Grant

The flavivirus E protein (which was described in the preceding abstract) adapts different conformations in immature and mature virus particles and in its low pH fusion competent configuration. The immature flavivirus particle contains 60 trimers of prM-E heterodimers. The prM protein covers the E protein, and protects it from undergoing premature fusion when passing through the acidic compartments and vesicles of the trans Golgi network. Following cleavage of prM by furin the virus enter its mature state in which the E proteins form 90 head-to-tail dimers that lie parallel to and completely cover the viral membrane. After entering cells by receptor-mediated endocytosis, fusion with the host membrane is initiated by a low pH-induced conformational change of the E protein. Thus, flexibility of the E protein is a functional requirement for both assembly and infection. Zhang *et al*. (*Structure*, Vol. 12, September, 2004) compared crystal structures of Dengue sE and the structures of the E monomer in the immature and mature virions and found that the E protein can bend about a hinge between domain II and domains I plus III. By fitting the E glycoprotein into the cryo-electron microscopy maps of both immature and mature dengue virus, they showed that during maturation DI+DIII reorients with respect to the viral membrane and the hinge angle changes. Similarly rearrangement of E into a fusion competent state involves an even larger hinge motion. We have mutated a number of conserved glycine and alanine residues in the hinge region, hoping to affect the flexibility of E without

completely destroying its conformation. These mutants are A54I (mutation of alanine at residue 54 to isoleucine), A54K, A187I, G279T, G279I, G279K, and A187K. All of these mutants are now in the full-length cDNA clone of yellow fever virus and have been confirmed by sequencing. RNA was transcribed from the mutants and used to transfect BHK cells. RNA replication was detected by immunofluorescence, and virus infectivity checked by plaque assay. Mutants containing the underlined changes were infectious, but produced less virus than wild-type yellow fever. Mutants containing the non-underlined mutations had undetectable infectivity, which may mean that the flexibility of the protein has been altered. For these latter three mutants, we need to determine whether non-infectious particles are being produced, and if so, to produce sufficient quantities of these particles for cryoelectron microscopic reconstructions.

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291. *In vitro* reactivation of immature dengue virus particles

Brian Blood

Flaviviruses produce two surface glycoproteins that are used in the assembly of virus particles. These proteins, called prM and E, initially form a heterodimer. At some point in the assembly of virions prM is cleaved by the cellular protease furin to produce M. E then rearranges to form homodimers in the mature virion. It is possible to produce immature virus particles that contain uncleaved prM by growing the virus in the presence of ammonium chloride. This lysosomotrophic agent causes the pH of endosomal vesicles through which virus is transported to rise, which in turn prevents cleavage of prM by furin. It is not known if these immature particles are true intermediates in virus assembly; it has been reported that noninfectious, prM-containing particles can be converted into infectious virions by treatment with furin. We have treated immature dengue virus (DEN) *in vitro* with furin after low pH treatment. Cleavage of prM to pr and M was detected by silver staining and Western blot assay using anti-serum against DEN prM. However, we could not detect M protein due to the small size of the protein and unavailability of antiserum to M. Reactivated infectivity was assayed by infecting BHK cells with furin-treated virions. Infected cells were detected by immunofluorescence using anti-DEN antiserum. We have been able to reactivate immature particles to form infectious particles. However, we were unable to detect the reactivated particles by electron microscopy. The ultimate goal is to reactivate sufficient numbers of particles for cryo-electron reconstructions. We have made progress in increasing the number of particles by fine tuning the pH of the furin reaction. We are also going to pursue increases in the amount of furin.

292. *In vitro* reconstitution of an enveloped animal virus

Odisse Azizgolshani, William M. Gelbart*, Charles M. Knobler*, Marlene Biller*

Sindbis virus (SINV) is an enveloped animal virus composed of a spherical nucleocapsid (NC) and a viral membrane. The NC is made of 240 identical copies of the capsid protein forming a shell around a positive sense RNA molecule. The NC is in turn enveloped by the membrane, which contains 240 heterodimers of spike proteins embedded in a lipid bilayer. The nucleocapsids are assembled in the cell cytoplasm and migrate to domains of cell plasmalemma containing the viral spikes. There the spike protein heterodimers interact one-to-one with the capsid proteins through hydrophobic bonds and as a final stage in the viral life cycle, the nucleocapsids bud out of the cell as mature, infectious virions. The goal of this project is to mimic *in vitro* this last stage of the SINV life cycle by wrapping viral membranes around naked nucleocapsids to form mature, infectious virions and to show in this way that budding is a thermodynamically favored event and that no active processes are required.

Our approach involves removing the envelope from intact virions using minimal amounts of a suitable non-denaturing detergent that preserves the integrity of the membrane and prevents loss of the spike proteins. We have found that such a detergent treatment leads to loss of infectivity, as measured by plaque assays. Currently, we are testing for the disassembly of the envelope from the NCs by running the detergent-treated samples on gradients to separate the NCs from envelopes by either sedimentation velocity or by equilibrium sedimentation. If the disassembly has occurred, envelope and NCs will be found in different fractions. Fractions containing the membranes and NCs will then be mixed together and allowed to interact again as the detergent is removed. We anticipate that reassembly will be facilitated by high concentrations of both components and by the gradual removal of the detergent. In preliminary experiments, detergent-treated virions (but not separated fractions) were loaded onto Centricon devices and the detergent was removed gradually by incremental addition of buffer. Under these conditions there was partial restoration of infectivity, suggesting that enveloped particles had reformed. We intend to confirm the formation of intact, enveloped virions by electron microscopy. Since NCs can be self-assembled *in vitro*, we also plan to synthesize NCs containing a reporter gene such as Luciferase or Green Fluorescent Protein within the SINV genome, under the control of a second subgenomic promoter. These *in vitro* assembled NCs will be added to the reassembly reactions in the Centricons. If infectious enveloped particles are formed, the reporter gene should be expressed in the plaques. This is an important control to prove that NCs and membranes had been completely separated before the reassembly process.

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Publication

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Summary: Our main subject is the ubiquitin system. The field of ubiquitin and regulated protein degradation was created in the 1980s, largely through the complementary discoveries by the laboratory of A. Hershko (Technion, Haifa, Israel) and by my laboratory, then at MIT. These discoveries revealed three sets of previously unknown facts:

1. That ATP-dependent protein degradation involves a new protein modification, ubiquitin conjugation, which is mediated by specific enzymes, termed E1, E2 and E3.
2. That the selectivity of ubiquitin conjugation is determined by specific degradation signals (degrons) in short-lived proteins, including the degrons that give rise to the N-end rule.
3. That ubiquitin-dependent processes play a strikingly broad, previously unsuspected part in cellular physiology, primarily by controlling the *in vivo* levels of specific proteins. Ubiquitin conjugation was demonstrated by us to be required for the protein degradation *in vivo*, for cell viability, and also, specifically, for the cell cycle, DNA repair, protein synthesis, transcriptional regulation, and stress responses. In addition, ubiquitin-dependent proteolysis was also discovered to involve a substrate-linked poly-Ub chain of unique topology that is required for protein degradation. The ubiquitin system was also discovered to possess subunit selectivity, i.e., the ability to destroy a specific subunit of a multisubunit protein, leaving the rest intact and thereby making possible *protein remodeling*.

The Hershko laboratory produced the first of these fundamental advances (item 1), and my laboratory produced the other two (items 2 and 3). Over the last 15 years, these complementary discoveries led to the enormous expansion of the ubiquitin field, which became one of the largest arenas in biomedical science, the point of convergence of many disparate disciplines. (For an account of the early history of the ubiquitin field, see Varshavsky, 2006). Our biological discoveries of the 1980s, together with later studies by many excellent laboratories that entered the field in the 1990's and afterward, yielded the modern paradigm of the central importance of regulated proteolysis for the control of the levels of specific proteins *in vivo*, as distinguished from their control by transcription and protein synthesis. In other words, these advances revealed that the control through regulated protein degradation rivals, and often surpasses in significance the classical regulation through transcription and translation. This radically changed understanding of the design of biological circuits will have (in fact, is already beginning to have) a major impact on medicine, given the astounding functional range of the ubiquitin system and the multitude of ways in which ubiquitin-dependent processes can malfunction in disease or in the course of aging, from cancer and neurodegenerative syndromes to perturbations of immunity and many other illnesses, including birth defects. Our work at Caltech continues to focus on ubiquitin-dependent processes.

The effect of an intracellular protein on the rest of the cell depends on the protein's concentration. The latter is determined by the rate of synthesis and/or import of the protein in relation to the rates of its degradation, inactivation by other means, or export from the compartment. The *in vivo* half-lives of intracellular proteins range from a few seconds to many days. Over the last decade, a vast number of biological circuits were shown to contain either constitutively or conditionally short-lived proteins. In addition, damaged or otherwise abnormal intracellular proteins tend to be recognized as such and selectively degraded, largely by the same ubiquitin-proteasome system that mediates the controlled proteolysis of short-lived regulatory proteins. The metabolic instability of a regulator provides a way to generate its spatial gradients and allows for rapid adjustments of its concentration (or subunit composition) through changes in the rate of its synthesis or degradation.

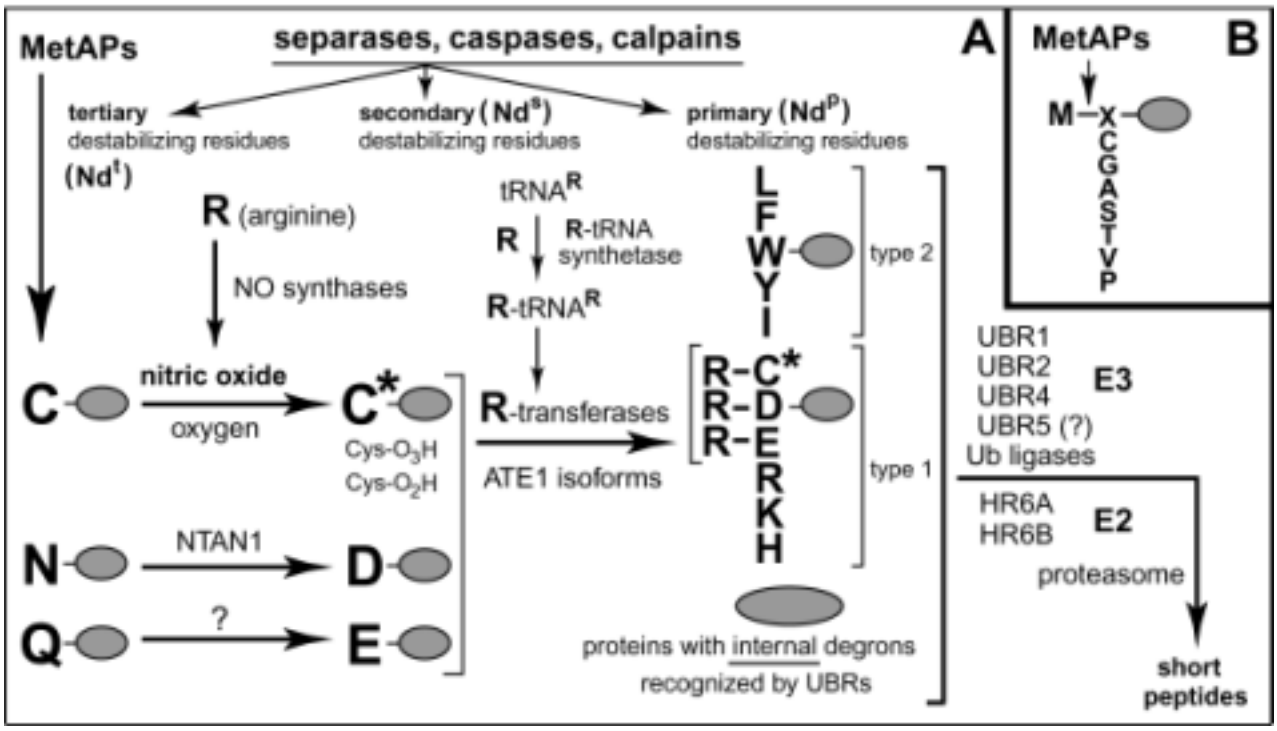

Fig. 1. (A) The N-end rule pathway in mammals. N-terminal residues are indicated by single-letter abbreviations for amino acids. The ovals denote the rest of a protein substrate. MetAPs, methionine aminopeptidases. The "cysteine" (Cys) sector, in the upper left corner, describes the discovery, by Hu *et al.* (2005), of the nitric oxide (NO)-mediated oxidation of N-terminal Cys, with subsequent arginylation of oxidized Cys by *ATE1*-encoded isoforms of Arg-tRNA-protein transferase (R-transferase). C* denotes oxidized Cys, either Cys-sulfinic acid ($CysO_2(H)$) or Cys-sulfonic acid ($CysO_3(H)$). Type 1 and type 2 primary destabilizing N-terminal residues are recognized by multiple E3 Ub ligases of the N-end rule pathway, including UBR1 and UBR2 (Tasaki *et al.*, 2005). Through their other substrate-binding sites, these E3s also recognize internal (non-N-terminal) degrons in other substrates of the N-end rule pathway, denoted by a larger yellow oval. **(B)** MetAPs remove Met from the N-terminus of a polypeptide if the residue at position 2 belongs to the set of residues shown.

Ubiquitin (Ub) is a 76-residue protein that exists in cells either free or conjugated to many other proteins. Degradation of intracellular proteins by the Ub-proteasome system regulates a multitude of processes: cell growth; cell division and differentiation; signal transduction; responses to stress; and a broad range of metacellular (organismal) processes, such as embryonic development, immunity and the functions of the nervous system. Ub-dependent proteolysis involves the "marking" of a substrate through covalent conjugation of Ub to a substrate's internal Lys residue. Ub conjugation is mediated by the E1-E2-E3 enzymatic cascade. E1, the ATP-dependent Ub-activating enzyme, forms a thioester bond between the C-terminal Gly of Ub and a specific Cys residue of E1. In the second step, activated Ub is transesterified to a Cys residue of a Ub-conjugating (E2) enzyme. Thereafter a complex of E2 and another enzyme, E3, conjugates Ub to a Lys residue of a substrate. The functions of E3 include the recognition of a substrate's degradation signal (degron). The numerous proteolytic pathways of the Ub system have in common their dependence on Ub conjugation and the 26S proteasome (which processively degrades Ub-protein conjugates), and differ largely through their utilization of distinct E2-E3 complexes. Specific E3s recognize (bind to) specific degrons of their substrates. The diversity of E3s and E2s (there are more than 500 distinct E3s in a mammal) underlies the enormous range of substrates that are recognized and destroyed by the Ub system, in ways that are regulated both temporally and spatially. Ub has nonproteolytic functions as well.

One pathway of the Ub system is the N-end rule pathway (Fig. 1). A specific class of degrons recognized by this pathway comprises degradation signals called N-degrons. In eukaryotes, an N-degron consists of three determinants: a destabilizing N-terminal residue of a protein substrate, its internal Lys (K) residue(s) (the site of formation of a poly-Ub chain);, and a conformationally flexible region(s) in the vicinity of these determinants that is required for the substrate's ubiquitylation and/or degradation. The set of destabilizing residues in a given cell type yields a rule, called the N-end rule, which relates the *in vivo* half-life of a protein to the identity of its N-terminal residue.

The N-end rule has a hierarchic structure (Fig. 1). In eukaryotes, N-terminal Asn (N) and Gln (Q) are tertiary destabilizing residues (denoted as Nd^t) in that they function through their enzymatic deamidation, to yield the secondary destabilizing N-terminal residues Asp (D) and Glu (E) (denoted as Nd^s). The destabilizing activity of N-terminal Asp and Glu requires their conjugation, by *ATE1*-encoded isoforms of Arg-tRNA-protein transferase

(arginyl-transferase or R-transferase), to Arg (R), one of the primary destabilizing residues (denoted as Nd^p). The latter N-terminal residues are recognized by E3 Ub ligases of the N-end rule pathway, called N-recognins. The N-end rule pathway of the yeast *Saccharomyces cerevisiae* is mediated by a single N-recognin, UBR1, while at least four N-recognins, including UBR1, mediate this pathway in mammals (Fig. 1) (Tasaki *et al.*, 2005).

An N-degron is produced from pre-N-degron through a proteolytic cleavage. Methionine aminopeptidases (MetAPs) remove Met from the N-terminus of a newly formed protein if the residue at position 2, to be made N-terminal after cleavage, has a small enough side chain. Consequently, amongst the 13 destabilizing residues of the mammalian N-end rule (Fig. 1A), only Cys can be made N-terminal by MetAPs (Fig. 1B). (Any destabilizing residue, including Cys, can be made N-terminal through internal cleavages of proteins by other proteases, such as separases, caspases, and calpains (Fig. 1A).) Our previous work showed that in mammals and other eukaryotes that produce nitric oxide (NO), the set of arginylated residues contains not only Asp and Glu, but also N-terminal Cys (C), which is arginylated after its oxidation (Kwon *et al.*, 2002). The *in vivo* oxidation of N-terminal Cys requires NO, as well as oxygen (O_2) or its derivatives (Fig. 1) (Hu *et al*., 2005).

Although prokaryotes lack Ub conjugation and Ub itself, they contain (Ub-independent) N-end rule pathways. The sets of destabilizing residues in prokaryotic N-end rules contain secondary destabilizing (Nd^s) residues as well. Their identities (Arg and Lys in *E. coli*; Arg, Lys, Asp and Glu in some other prokaryotes) are either overlapping with, or distinct from, the eukaryotic Nd^s residues (Graciet *et al.*, 2006). The activity of Nd^s residues in prokaryotes requires their conjugation to Leu (an Nd^p residue) by Leu-tRNA-protein transferases (L-transferases), in contrast to the conjugation of Arg to N-terminal Asp, Glu, or (oxidized) Cys in eukaryotes (Fig. 1). Prokaryotic L-transferases are of two distinct kinds, differing in both amino acid sequence and substrate specificity. Remarkably, L-transferases of one class, encoded by *bpt* genes, are sequelogs of *ATE1*-encoded eukaryotic R-transferases, despite the fact that Bpt (prokaryotic) aminoacyl-transferases conjugate Leu, rather than Arg, to the N-termini of cognate substrates (Graciet *et al*., 2006).

In this terminology (Varshavsky, 2004), "sequelog" and "spalog" denote, respectively, a sequence that is similar, to a specified extent, to another sequence, and a 3D structure that is similar, to a specified extent, to another 3D structure. "Sequelog" and "spalog" are mnemonically helpful, single-word terms whose rigor-conferring advantage is their *evolutionary neutrality*. The sequelog terminology conveys the fact of sequence similarity (sequelogy) without evolutionary or functional connotations, in contrast to interpretation-laden terms such as homolog, ortholog and paralog. The latter terms are compatible with the sequelog/spalog terminology and can be employed to convey understanding about functions and common descent, if this information is actually available (Varshavsky, 2004).]

The functions of the N-end rule pathway (see also Abstracts below) include the regulation of import of short peptides (through the degradation, modulated by peptides, of the import's repressor), the fidelity of chromosome segregation (through the degradation of a conditionally produced cohesin fragment), the regulation of apoptosis (through the degradation of a caspase-processed inhibitor of apoptosis), the regulation of meiosis, the leaf senescence in plants, as well as neurogenesis and cardiovascular development in mammals. M. Zenker and colleagues have discovered, in a collaboration that included our lab as well, that mutations in human UBR1, one of several functionally overlapping N-recognins (Fig. 1), are the cause of Johanson-Blizzard Syndrome (JBS), which comprises mental retardation, physical malformations, and severe pancreatitis (Zenker *et al.*, 2005). Abnormalities of the previously constructed $UBR1^{-/-}$ mouse strains (Kwon *et al*., 2001) include pancreatic insufficiency, a less severe version of the defect in human JBS ($UBR1^{-/-}$) patients (Zenker *et al*., 2005). The cardiovascular and (probably) other functions of the N-end rule pathway involve the arginylation-mediated degradation of RGS4, RGS5, and RGS16 (Hu *et al*., 2005; Lee *et al.*, 2005). These "GTPase-activating" proteins function by inhibiting the signaling by specific G proteins, and are themselves down-regulated through the NO/O_2-dependent degradation by the N-end rule pathway (Hu *et al.*, 2005). The N-terminal Cys residues of RGS4, RGS5 and RGS16 are oxidized *in vivo* at rates controlled by NO and oxygen, followed by the arginylation of oxidized Cys and processive proteolysis by the rest of the N-end rule pathway (Fig. 1). The arginylation branch of this pathway is also essential for chromosome stability, through its targeting and selective destruction of a separase-produced C-terminal fragment of a cohesin's subunit that bears a destabilizing N-terminal residue (Rao *et al.*, 2001). While the identity of the key residue that becomes N-terminal upon cleavage by separase varies amongst cohesins of different eukaryotes, it is a destabilizing residue in all eukaryotes examined, indicating the importance of maintaining, in evolution, a short *in vivo* half-life of the cohesin fragment. In particular, the N-end rule pathway is also required for degradation of the *in vivo*-produced fragment of the mammalian RAD21/SCC1 subunit of cohesin (J. Zhou, J. Sheng, R.-G. Hu and A. Varshavsky, unpublished data).

Functional and mechanistic studies of the N-end rule pathway in yeast and mammals are a major theme of our current work.

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293. The N-end rule pathway as a nitric oxide sensor controlling the levels of multiple regulators

Cory Hu, Jun Sheng, Xin Qi, Zhenming Xu, Terry Takahashi, Alexander Varshavsky

Nitric oxide (NO) is produced in eukaryotes largely by NO synthases. This compound and its derivatives play a role, as either stressors or regulators, in a vast range of functions, including cardiovascular homeostasis, immunity, neurotransmission, ion conductance, glycolysis, and apoptosis. Biological effects of NO are mediated by its covalent modifications of proteins, either of their prosthetic groups or amino acid residues, particularly Cys and Tyr. The reactivity of these residues toward NO is modulated by their sequence contexts. NO converts Cys residues to S-nitrosothiols, a process that can involve oxygen or its derivatives. S-nitrosylation modulates protein functions either directly or after additional (often oxygen-dependent) transformations that yield oxidized Cys, such as Cys-sulphinic acid ($CysO_2(H)$) or Cys-sulphonic acid ($CysO_3(H)$).

The lab's previous work has shown that in mammals destabilizing N-terminal residues which function through their arginylation are not only Asp and Glu but also Cys, which is a stabilizing (unarginylated) residue in the yeast *S. cerevisiae* (Fig. 1A) (Gonda *et al.*, 1989; Kwon *et al.*, 2002).

In 2005, we demonstrated that the oxidation of N-terminal cysteine is essential for its arginylation (Hu *et al.*, 2005). Most importantly, it was also discovered that the *in vivo* oxidation of N-terminal cysteine, prior to its arginylation, require nitric oxide (NO) (Fig. 1) (Hu *et al.*, 2005). This accounted for N-terminal Cys being a destabilizing residue in mammalian cells, which produce NO, but stabilizing in yeast, which lack NO synthases. Thus, the N-end rule pathway is a new kind of NO sensor in mammalian cells, functioning through its ability to destroy specific regulatory proteins bearing N-terminal Cys residue, at rates controlled by NO, and by oxygen as well.

The levels of regulatory proteins with a specific N-terminal motif [Cys-(basic residue)], such as RGS4, RGS5 and RGS16, were found to be greatly increased in mouse *ATE1(/)* embryos, which lack arginylation (Hu *et a*l., 2005). Given the known involvement of these RGS proteins in the control of heart functions, angiogenesis, and other tubulogenesis pathways, the demonstrated stabilization of these RGSs in *ATE1(/)* embryos is likely to underlie the previous findings by our lab of abnormal angiogenesis and heart defects in the absence of arginylation (Kwon *et al.*, 2002). RGS4, RGS5, and RGS16 down-regulate signaling by specific G proteins, through acceleration of GTP hydrolysis by their G-alpha subunits. The N-end rule pathway thus, regulates signaling by G-protein-coupled receptors, through the conditional (NO-dependent) destruction of RGS4, RGS5 and RGS16 (Hu *et al.*, 2005; Lee *et al.*, 2005). Our study (Hu *et al.*, 2005) has also revealed other NO-dependent N-end rule substrates that are encoded by the mouse (and human) genome. Specifically, a mammalian genome encodes approximately 30 proteins, including RGS4, RGS5 and RGS16, that contain the Met-Cys-[basic residue] N-terminal motif, which acts as a MetAP-cleaved, NO-dependent, arginylation-mediated, Cys-containing pre-N-degron. Together, the above discovery identified the arginylation branch of the N-end rule pathway as a new sensor of NO in mammalian cells (Hu *et al.*, 2005). The resulting "unification" of a ubiquitin-dependent proteolytic pathway and the signaling by NO uncovered a new mechanism of NO signaling in the heart and other organs: the modulation of activity of regulatory proteins bearing N-terminal Cys through their NO-dependent, arginylation-mediated degradation by the N-end rule pathway. One medical ramification of this discovery is that pharmacological manipulation of arginyl-transferases is likely to yield a more selective route to beneficial effects that are currently achieved using drugs that alter the levels of NO [62].

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294. Arginyl-transferase: Specificity, putative substrates, bidirectional promoter, and splicing-derived isoforms

Rong Gui Hu, Christopher Brower, Haiqing Wang, Ilia V. Davydov[1], Jun Sheng, Jianmin Zhou, Yong Tae Kwon[2], and Alexander Varshavsky

Substrates of the N-end rule pathway include proteins with destabilizing N-terminal residues. Three of them – Asp, Glu and (oxidized) Cys – function through their conjugation to Arg, one of destabilizing N-terminal residues that are recognized directly by the pathway's ubiquitin ligases. The conjugation of Arg is mediated by arginyl-transferase, encoded by *ATE1*. Through its regulated degradation of specific proteins, the arginylation branch of the N-end rule pathway mediates, in particular, the cardiovascular development, the fidelity of chromosome segregation, and the control of signaling by nitric oxide (see Introduction, Fig. 1, Abstract 1, and Hu *et*

al., 2005). We found that mouse *ATE1* specifies at least six mRNA isoforms, which are produced through alternative splicing, encode enzymatically active arginyl-transferases, and are expressed at varying levels in mouse tissues. We also found that the *ATE1* promoter is bidirectional, mediating the expression of both *ATE1* and an oppositely oriented, previously uncharacterized gene. In addition, we identified glucose-regulated protein 78 (GRP78) and protein disulfide isomerase (PDI) as putative physiological substrates of arginyl-transferase. Purified isoforms of arginyl-transferase that contain the alternative first exons differentially arginylate these proteins in extract from *ATE1*$^{-/-}$ embryos, suggesting that specific isoforms may have distinct functions. While the N-end rule pathway is apparently confined to the cytosol and the nucleus, and although GRP78 and PDI are located largely in the endoplasmic reticulum, recent evidence suggests that these proteins are also present in the cytosol and other compartments *in vivo*, where they may become N-end rule substrates.

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295. Mechanistic and functional studies of N-terminal arginylation

Christopher Brower, Jianmin Zhou, Rong Gui Hu, Haiqing Wang, Jun Sheng, Alexander Varshavsky

This Abstract mentions some of our ongoing projects that aim to further advance the understanding of the arginylation branch of the N-end rule pathway, beyond the recent advances that are described in Abstracts 1 and 2.

(**i**) Construction and functional analyses of mouse strains (and cells derived from them) in which the expression of *ATE1*-encoded Arg-tRNA-protein transferases (R-transferases) is selectively and conditionally abolished in specific cell lineages during embryogenesis, or postnatally (*Christopher Brower*). This set of projects should make possible, among other things, a functional dissection of N-terminal arginylation in specific organ systems and cell types of adult mice. (A nonconditional *ATE*$^{-/-}$ genotype is embryonic lethal (Kwon *et al*., 2002).

(**ii**) Construction and functional analysis of knock-in mouse strains that contain a doxycycline-inducible allele of *ATE1*, and thus, can overproduce R-transferases, in a controllable manner, in specific cell types during embryogenesis, or postnatally (*Jianmin Zhou, Christopher Brower*).

(iii) Analysis of chromosome stability and regulation of apoptosis in mouse *ATE1*$^{-/-}$ cells (*Jianmin Zhou, Jun Sheng, Cory Hu, and Christopher Brower*). These projects stem from the discovery of the function of the *S. cerevisiae* N-end rule pathway in the maintenance of chromosome stability (Rao *et al*., 2001), and from the conjecture that an analogous function in mammalian cells involves the (*ATE1*-dependent) arginylation branch of the N-end rule pathway. Recent work indicated that N-terminal arginylation is essential for the *in vivo* degradation of the separase-produced fragment of SCC1/RAD21, a subunit of mouse cohesin. Moreover, *ATE1(*$^{-/-}$*)* fibroblasts were also found exhibit a strong chromosome instability (CIN), presumably because, at least in part, of their inability to destroy the separase-produced cohesin's fragment (*Jianmin Zhou*).

(iv) Identification of *ATE1*-dependent circuits (i.e., circuits that involve N-terminal arginylation) through the identification of mouse genes whose expression is significantly altered during embryonic development in *ATE*$^{-/-}$ embryos, using microarray techniques, differential display and analogous methods with *ATE(*$^{-/-}$*)* and congenic +/+ embryos or EF cells (*Cory Hu, Jun Sheng, Jianmin Zhou*).

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296. New aminoacyl-transferases and the N-end rule pathway of prokaryotic/eukaryotic specificity in a human pathogen

Emmanuelle Graciet, Rong Gui Hu, Konstantin Piatkov, Joon Haeng Rhee[1]*, Erich Schwarz, Alexander Varshavsky*

The N-end rule relates the *in vivo* half-life of a protein to the identity of its N-terminal residue (see Introduction). Primary destabilizing N-terminal residues (Nd^p) are recognized directly by the targeting machinery. The recognition of secondary destabilizing N-terminal residues (Nd^s) is preceded by conjugation of an Nd^p residue to Nd^s of a polypeptide substrate. In eukaryotes, *ATE1*-encoded arginyl-transferases (R^{D,E,C^*}-transferases) conjugate Arg (R), an Nd^p residue, to Nd^s residues Asp (D), Glu (E) or oxidized Cys (C*) (Fig. 1). Ub ligases recognize the N-terminal Arg of a substrate and target the (ubiquitylated) substrate to the proteasome. In prokaryotes such as *Escherichia coli*, Nd^p residues Leu (L) or Phe (F) are conjugated, by the *aat*-encoded Leu/Phe-transferase ($L/F^{K,R}$-transferase), to N-terminal Arg or Lys, which are Nd^s in prokaryotes but Nd^p in eukaryotes. In prokaryotes, substrates bearing the Nd^p residues Leu, Phe, Trp or Tyr are degraded by the proteasome-like ClpAP protease (Tobias *et al*., 1991; Shrader *et al*., 1993).

Despite enzymological similarities between eukaryotic R^{D,E,C^*}-transferases and prokaryotic $L/F^{K,R}$-transferases, there is no significant sequelogy (sequence similarity) between them (see a brief description, in square parentheses in Introduction, of the recently introduced terms "sequelog" and "spalog) (Varshavsky, 2004). We have identified a new aminoacyl-transferase, termed Bpt, in the human pathogen *Vibrio vulnificus* (Graciet *et al.*, 2006). Although it is a sequelog of eukaryotic R^{D,E,C^*}-transferases, this prokaryotic transferase was found to exhibit a "hybrid" specificity, conjugating Nd^p Leu to Nd^s Asp or Glu, the first such Nd^s residues in prokaryotes. Another new aminoacyl-transferase, termed ATEL1, of the eukaryotic pathogen *Plasmodium falciparum*, is a sequelog of prokaryotic $L/F^{K,R}$-transferases (Aat), but has the specificity of eukaryotic R^{D,E,C^*}-transferases (ATE1) (Graciet *et al.*, 2006). In addition, our phylogenetic analysis suggests that the substrate specificity of R-transferases arose by two distinct routes during the evolution of eukaryotes (Graciet al., 2006).

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297. Construction and analysis of mouse strains lacking the UBR3 ubiquitin ligase

Takafumi Tasaki[1], Yong Tae Kwon[1], Alexander Varshavsky

Kwon *et al.* (1998) identified two distinct mouse (and human) genes, termed *UBR2* and *UBR3*, which encode proteins that are similar to mouse and yeast UBR1, the first E3 of the N-end rule pathway to be characterized. (It was also the first Ub ligase of the entire Ub system to be identified.) In contrast to the highly similar mouse UBR1 and UBR2 proteins (47% identity and 68% similarity), the mouse UBR3 protein, while clearly a member of the UBR family, is less similar to UBR1 (25% identity and 51% similarity) and UBR2 (25% identity and 48% similarity). In addition, mouse UBR3 lacks some of the residues in its N-terminal region that have been shown to be essential for the function of yeast UBR1, and are also present in the mouse (and human) UBR1 and UBR2 proteins. We mapped and partially sequenced the mouse *UBR3* gene, and more recently constructed *UBR3(/)* mouse strains. *UBR3(/)* mice are viable, and are being characterized.

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298. Quantitative analyses of interactions between components of the N-end rule pathway and their substrates or effectors

Zanxian Xia, Alexander Varshavsky

Detailed understanding of the N-end rule pathway requires, among other things, the knowledge of equilibrium binding constants for the reversible interactions between UBR1 and its effectors or substrates. (With some interactions, it would be desirable to know the corresponding rate constants, as well.) We are carrying out these measurements using the fluorescence polarization (FP) technique, with purified *S. cerevisiae* UBR1 (or its fragments) and a variety of UBR1 ligands, including peptides with destabilizing N-terminal residues. These analyses will be expanded to include other physiological ligands of UBR1 such as the RAD6 Ub-conjugating enzyme, and CUP9, a homeodomain repressor recognized through its C-terminal degron by a distinct (third) substrate-binding site of UBR1.

299. Phosphorylation of UBR1: Its regulation and functions

Cheol Sang Hwang, Zanxian Xia, Alexander Varshavsky

S. cerevisiae UBR1, the E3 of the yeast N-end rule pathway, is phosphorylated *in vivo*, but the role(s) of this UBR1 modification in the multiple functions of the N-end rule pathway is unknown. Phosphorylation sites on UBR1 and the kinases/phosphatases involved remain to be identified, as well. We are using biochemical and genetic approaches to understand, in functional and mechanistic detail, this modification of UBR1.

300. A family of mammalian E3 ubiquitin ligases that contain the UBR motif and recognize N-degrons

Takafumi Tasaki[1], Lubbertus Mulder[2], Akihiro Iwamatsu[3], Min Jae Lee[1], Ilia Davydov[4], Alexander Varshavsky, Mark Muesing[2], Yong Tae Kwon[1]

Substrates of the N-end rule pathway include proteins that contain degradation signals called N-degrons (Fig. 1A). One determinant of N-degron is a substrate's destabilizing N-terminal residue. Previous work (Kwon *et al*., 1998; Kwon *et al*., 2001; Kwon *et al*., 2003) identified mammalian UBR1 and UBR2 as E3 Ub ligases that recognize N-degrons. We report here that while *UBR1 / UBR2 /* mice died as early embryos, the rescued *UBR1 / UBR2 /* fibroblasts still had the N-end rule pathway. An affinity assay for proteins that bind to destabilizing N-terminal residues has identified, in addition to UBR1 and UBR2, a huge (570 kDa) mouse protein, termed UBR4, and also the 300-kDa UBR5, a previously characterized mammalian E3 known as EDD/hHYD. A counterpart of mammalian UBR4 is known as PUSHOVER in *Drosophila* and BIG in plants.

UBR1 / UBR2 / fibroblasts that were made also deficient in UBR4 were impaired in the degradation of N-end rule substrates, confirming that UBR4 is a part of the N-end rule pathway. The recognition of destabilizing N-terminal residues by UBR4 and UBR5 is a new property of these proteins. UBR1, UBR2, UBR4, UBR5, and at least three other mouse proteins contain a motif termed the UBR box. The resulting UBR family consists of either demonstrated or inferred Ub ligases. A major part of this family are E3s of the N-end rule pathway. Thus, in contrast to the yeast *S. cerevisiae*, where the N-end rule pathway is dependent on just one Ub ligase, UBR1, a mammalian N-end rule pathway is mediated by at least four distinct Ub ligases, which have in common at least the UBR box.

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301. Deficiency of UBR1, a ubiquitin ligase of the N-end rule pathway, causes pancreatic dysfunction, malformations and mental retardation (Johanson-Blizzard syndrome)

Martin Zenker[1], Julia Mayerle, Markus M. Lerch*, Andreas Tagariello*, Klaus Zerres*, Peter R. Durie*, Matthias Beier*, Georg Hülskamp*, Celina Guzman*, Helga Rehder*, Frits A. Beemer*, Ben Hame[1], Philippe Vanlieferinghen*, Ruth Gershoni Baruch*, Marta W. Vieira*, Miroslav Dumic*, Ron Auslender*, Vera L. Gil da Silva Lopes*, Simone Steinlicht*, Manfred Rauh*, Stavit A. Shalev*, Christian Thiel[1], Andreas Winterpacht[1], Yong Tae Kwon[2], Alexander Varshavsky, and André Reis[1]*

Johanson-Blizzard syndrome is an autosomal recessive disorder that includes congenital exocrine pancreatic insufficiency, multiple malformations, such as nasal wing aplasia, and frequent mental retardation. A multi-laboratory collaboration led by M. Zenker (Germany) that involved this lab as well mapped the disease locus to chromosome 15q14-21.1. Zenker *et al*. (2005) identified mutations, mostly truncating ones, in the *UBR1* gene in 12 unrelated JBS families. As described in Introduction, *UBR1* encodes one of at least four functionally overlapping E3 ubiquitin ligases of the N-end rule pathway, a conserved proteolytic system whose substrates include proteins with destabilizing N-terminal residues (Fig. 1). Pancreas of JBS patients did not express UBR1, and exhibited intrauterine-onset destructive pancreatitis. We also show that UBR1–/– mice, whose previously reported phenotypes include reduced weight and behavioral abnormalities, have an exocrine pancreatic insufficiency, with impaired stimulus-secretion coupling and increased susceptibility to pancreatic injury. These findings (Zenker *et al.*, 2005) indicate that deficiency of *UBR1* perturbs pancreatic acinar cells and other organs, presumably owing to metabolic stabilization of specific substrates of the N-end rule pathway.

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Developmental and Regulatory Biology

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Summary: This laboratory's research centers on the early formation of the nervous system in vertebrate embryos. The peripheral nervous system forms from two cell types that are unique to vertebrates: neural crest cells and ectodermal placodes. We study the cellular and molecular events underlying the formation, cell lineage decisions and migration of these two cells type. The neural crest is comprised of multipotent stem cell-like precursor cells that migrate extensively and give rise to an amazingly diverse set of derivatives. In addition to their specific neuronal and glial derivatives, neural crest cells can also form melanocytes, craniofacial bone and cartilage and smooth muscle. Placodes are discrete regions of thickened epithelium that give rise to portions of the cranial sensory ganglia as well as form the paired sense organs (lens, nose, ears). Placodes and neural crest cells share several properties including the ability to migrate and to undergo an epithelial to mesenchymal transition. Their progeny are also similar: sensory neurons, glia, neuroendocrine cells, and cells that can secrete special extracellular matrices.

Our laboratory concentrates on studying the cellular and molecular mechanisms underlying the induction, early development and evolution of the neural crest and placodes. This research addresses fundamental questions concerning cell commitment, migration and differentiation using a combination of techniques ranging from experimental embryology to genomic approaches to novel gene discovery and identification of gene regulatory regions. These studies shed important light on the mechanisms of neural crest and placode formation, migration and differentiation. In addition, the neural crest and placodes are unique to vertebrates. In studying the evolution of these traits, we hope to better understand the origin of vertebrates.

Because these cell types are involved in a variety of birth defects and cancers such as neurofibromatosis, melanoma, neuroblastoma, our results on the normal mechanisms of neural crest development provide important clues regarding the mistakes that may lead to abnormal development or loss of the differentiated state.

302. Importance of neural crest in development and evolution of the pharynx

David W. McCauley, Marianne Bronner Fraser*

Neural crest cells are a defining character of vertebrates and of prime importance in the evolutionary origin of the vertebrate body plan. To understand neural crest evolution, we explored molecular mechanisms underlying craniofacial development in the basal jawless vertebrate, lamprey, focusing on genes of the SoxE (Sox8, Sox9, and Sox10) family. In jawed vertebrates, these have been shown to be important transcriptional regulators of neural crest development and loss of Sox9 specifically causes abnormal cartilage formation. Here we show that a lamprey SoxE gene, *PmSoxE1,* is expressed in migrating neural crest cells and crest-derived prechondrocytes of the branchial arches. Furthermore, morpholino-induced protein knock down revealed that it is essential for branchial arch development. Thus, function of this transcriptional regulator can be traced to the lamprey-gnathostome common ancestor, suggesting that a SoxE gene was important in the evolution of vertebrate branchial arches and chondrogenesis. Our findings renew questions regarding the homology of vertebrate branchial arches.

**Assistant Professor, University of Oklahoma (former postdoc)*

303. Specification of neural crest occurs during gastrulation and requires Pax7

Martín L. Basch, Marianne Bronner Fraser, Martín I. García Castro***

The neural crest is a stem population critical for development of vertebrate craniofacial skeleton and peripheral ganglia. Classically, neural crest has been thought to form by interactions at the border between neural and non-neural ectoderm. In contrast, we report that neural crest induction is underway before proper neural plate appearance, during gastrulation. We show that a restricted region of stages 3 and 4 chick epiblast is specified to generate neural crest cells when explanted under non-inducing conditions. This same region of the gastrula begins to express the transcription factor Pax7 at stage 4+ and later contributes to neural folds and migrating neural crest, both of which express Pax7. Finally we show that Pax7 is required for neural crest formation *in vivo*, such that preventing Pax7 translation inhibits expression of the neural crest markers Slug, Sox 9, Sox10 and HNK-1. Our results suggest that neural crest specification initiates

earlier than previously assumed and that Pax7 plays a critical role during neural crest development.

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304. Neuropilin-2/semaphorin3F signaling is essential for segmental neural crest migration through the somites

Laura S. Gammill, Constanza Gonzalez, Marianne Bronner Fraser

Neural crest cells migrate in a segmental fashion in vertebrate embryos. We demonstrate that migrating neural crest cells express the receptor neuropilin-2 (npn-2), and that mice mutant for this receptor exhibit a variety of defects during neural crest migration. In the trunk, neural crest cells normally invade the rostral but not caudal half of each somitic sclerotome. In npn-2 nulls, neural crest cells are evenly distributed throughout the sclerotome, resulting in the loss of segmental migration. The repulsive npn-2 ligand semaphorin3F (sema3F) is selectively expressed in the caudal sclerotome, and sema3F null mice exhibit an identical phenotype to that of npn-2 mutants. At cranial levels, npn-2 mutant mice have loosely condensed trigeminal ganglia and "bridges" of migrating cells crossing between neural crest streams entering branchial arches 1 and 2. The results definitively show that npn-2/sema3F signaling directs neural crest migration, including rostral-only migration of neural crest through the somite. Interestingly, dorsal root ganglia still condense in a segmental pattern in npn-2 mutant mice, apparently sorting out from the uniform sheet of migrating neural crest, suggesting that segmental neural crest migration may not be essential for the formation of individualized ganglia.

305. The α5 subunit of laminin is required for proper murine neural crest cell migration

Edward G. Coles, Laura S. Gammill, Jeffery H. Miner, Marianne Bronner Fraser

Although numerous *in vitro* experiments suggest that extracellular matrix molecules like laminin can influence the neural crest migration, little is known about their function in cell migration in the embryo. We previously identified the α5 subunit of laminin in a screen for genes upregulated in response to neural crest induction. Here, we show that *laminin α5* transcripts are localized in regions of the newly formed cranial and trunk neural folds as well as adjacent to neural crest migratory pathways. The expression pattern is largely conserved between chick and mouse. In *laminin α5* mutant mice, neural crest migratory streams appear expanded compared to wild-type mice. Conversely, growing neural crest cells in the presence of laminin α5 subunit *in vitro* results in a reduction of migratory cells by about half. During neurogenesis, *laminin α5* mutant embryos exhibit defects in the condensing cranial sensory and trunk sympathetic ganglia. However, the ganglia are not totally missing and at later stages apparently recover. These data suggest that the *laminin α5* subunit, as a component in the extracellular matrix, acts as a non-permissive cue and is essential for proper migration and timely differentiation of some neural crest populations.

306. Neural crest-derived corneal keratocytes retain progenitor cell properties

Peter Lwigale, Paola Cressy, Marianne Bronner Fraser

Cranial neural crest cells migrate into the periocular region of the eye and contribute to the cornea. Upon reaching the presumptive corneal stroma, neural crest cells differentiate, synthesize extracellular matrix and become entrapped. We investigated whether corneal stromal keratocytes could dedifferentiate and contribute to neural crest derivatives when challenged with a new environment by injecting them into neural crest migratory pathways. Stromal keratocytes were enzymatically isolated from quail corneas, as a single cell suspension and then injected alongside the dorsal neural tube of stage 9 chick embryos. Analysis of embryos at various stages of development revealed that the injected stromal keratocytes dedifferentiated, proliferated and migrated ventrally adjacent to host neural crest cells. Injected stromal keratocytes contributed to the corneal endothelial and stromal layers, muscular of the mandibular process, blood vessels and cardiac cushion tissue. However, they failed to form neurons in cranial ganglia and branchial arch cartilage. Our data indicate that stromal keratocytes can migrate, dedifferentiate and contribute to the non-neuronal cranial neural crest derivatives. These results suggest that corneal stromal keratocytes even at late embryonic stages are not terminally differentiated, but rather maintain plasticity and multipotentiality.

307. Dynamic alterations in gene expression during avian neural crest induction

Lisa A. Taneyhill, Marianne Bronner Fraser

The Wnt signaling pathway is important in the formation of neural crest cells in many vertebrates. Because the downstream targets of induction are largely unknown, we have examined gene expression regulated by Wnt-mediated neural crest induction using quantitative polymerase chain reaction (QPCR). Neural crest induction was recapitulated *in vitro* by adding soluble Wnt to intermediate neural plate tissue cultured in collagen, and induced versus control tissue were assayed using gene-specific primers at times corresponding to premigratory (18 and 24 hours) or early (36 hours) stages of crest migration. The results show that Wnt signaling up regulates the expression of several genes normally expressed in the dorsal neural tube (*slug*, *Pax3*, *Msx1*, *FoxD3, cadherin 6B*) at "premigratory" stages. While *slug* is maintained in early migrating crest cells, *Pax3*, *FoxD3, Msx1* and *cadherin 6B* all are downregulated by the start of migration. These results contrast somewhat to the temporal expression of these genes in response to the addition of recombinant BMP4. Interestingly, expression of *rhoB* is unchanged or even decreased in response to

Wnt-mediated induction at all times examined, though it is upregulated by BMP signals. The temporal QPCR profiles in our culture paradigm approximate *in vivo* expression patterns of these genes prior to neural crest migration, but suggest that additional signals maintain these genes *in vivo*. Our results are the first to quantitatively describe changes in gene expression in response to a Wnt or BMP signal during transformation of a neural tube cell into a migratory neural crest cell.

308. Axial patterning in cephalochordates and the evolutionary origin of the gastrula organizer

Jr Kai Yu, Marianne Bronner Fraser, Nick Holland, Linda Holland

The organizer of the vertebrate gastrula, which induces and patterns dorsal axial structures, expresses a characteristic suite of transcription factors and secretes antagonists of BMP-, Nodal- and Wnt-signaling pathways. Despite long-standing interest, the evolutionary origin of the organizer remains unclear. Here we show that the gastrula of the cephalochordate amphioxus expresses orthologues of organizer genes in patterns reminiscent of their deployment in vertebrates. Genes involved in dorso/ventral (D/V) patterning include a dorsally expressed group (*Nodal, Lefty, Chordin,* and ADMP) as well as a ventrally expressed group (including BAMBI and Tolloid, which are BMP signaling modulators). Key transcription factors for dorsal/ventral (D/V) patterning also are expressed similarly to their vertebrate orthologues. Genes involved in anterior/posterior (A/P) patterning include a suite of *Wnt* genes expressed posteriorly around the blastopore and several Wnt antagonists, most expressed anteriorly, consistent with a role for *Wnts* in A/P patterning. These findings suggest that both cephalochordates and vertebrates use homologous gene networks for both D/V and A/P patterning. In light of recent phylogenetic analyses placing cephalochordates basal in the chordate lineage, it is likely that separate D/V and A/P signaling centers, as proposed for chick and mouse, may already have been present in ancestral chordates.

309. Neural crest gene regulatory network is evolutionarily conserved in a jawless basal vertebrate

Tatjana Sauka Spengler, Daniel Meulemans, Matthew Jones, Marianne Bronner Fraser

The neural crest is an embryonic cell population that is unique to vertebrates, forming much of the peripheral nervous system, facial skeleton, and body pigmentation. To elucidate the evolutionary history of the gene network driving neural crest development, we used moderate-throughput transcriptome analysis to identify homologues of neural crest regulatory genes from the basal jawless vertebrate, lamprey. We have isolated and examined the expression of 24 lamprey homologues of genes that function in gnathostome neural crest development. The roles of seven of these factors in lamprey neural crest cells were tested using a morpholino-mediated loss-of-function approach. We find a striking conservation not only of gene expression patterns, but also of function for nearly every gene analyzed, suggesting that the complete neural crest gene regulatory network arose early in vertebrate evolution and has been tightly conserved for over 500 million years.

310. Snail2 directly represses *cadherin6B* during epithelial-to-mesenchymal transitions of the neural crest

Lisa A. Taneyhill, Edward G. Coles, Marianne Bronner Fraser

The neural crest, a transient migratory population of cells, forms craniofacial skeleton, peripheral nervous system, and other derivatives. The transcriptional repressor Snail2 is thought to be critical for the epithelial-to-mesenchymal transition that promotes neural crest delamination from the neural tube; however, little is known about its downstream targets. To this end, we depleted avian Snail2 protein in the premigratory neural crest using morpholino antisense oligonucleotides and examined effects on potential targets by quantitative PCR. Several dorsal neural tube genes were upregulated by alleviation of Snail2 repression; moreover, the cell adhesion molecule *cadherin6B* was de-repressed as early as 30 minutes. *Cadherin6B* has a reciprocal expression pattern to Snail2, and its regulatory region contains three pairs of clustered E boxes, representing putative Snail2 binding sites. Furthermore, *in vivo* and *in vitro* biochemical analyses demonstrate that Snail2 directly binds to these sites. These results are the first to describe a direct target of Snail2 repression *in vivo* during the epithelial-to-mesenchymal transition that characterizes neural crest development.

311. Neuropilin 2/semaphorin 3F signaling is essential for cranial neural crest migration and trigeminal ganglion condensation

Laura S. Gammill, Constanza Gonzalez, Marianne Bronner Fraser

In the head of vertebrate embryos, neural crest cells migrate from the neural tube into the presumptive facial region and condense to form cranial ganglia and skeletal elements in the branchial arches. We show that newly formed neural folds and migrating neural crest cells express the *neuropilin 2* (*npn2*) receptor in a manner that is highly conserved in amniotes. The repulsive npn2 ligand *semaphorin (sema) 3F* is expressed in a complementary pattern in the mouse. Furthermore, mice carrying null mutations for either *npn2* or *sema3F* have abnormal cranial neural crest migration. Most notably, "bridges" of migrating cells were observed crossing between neural crest streams entering branchial arches 1 and 2. In addition, trigeminal ganglia fail to form correctly in the mutants and are improperly condensed and loosely organized. These data show that npn2/sema3F signaling is required for proper cranial neural crest development in the head.

312. Lens is the ground state of all sensory placodes, from which FGF promotes olfactory identity

Andrew P. Bailey, Sujata Bhattacharyya, Marianne Bronner Fraser, Andrea Streit

The sense organs of the vertebrate head comprise structures as varied as the eye, inner ear and olfactory epithelium. In the early embryo, these assorted structures share a common developmental origin within the pre-placodal region and acquire specific characteristics only later. Here we demonstrate for the first time a fundamental similarity in placodal precursors: all are specified as lens prior to acquiring features of specific sensory or neurogenic placodes. Lens specification becomes progressively restricted in the head ectoderm, initially by FGF and subsequently by signals derived from migrating neural crest cells. We show that FGF8 from the anterior neural ridge is both necessary and sufficient to promote olfactory fate in adjacent ectoderm. Our results reveal that placode precursors share a common ground state as lens and progressive restriction allows the full range of placodal derivatives to form.

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Summary: The major focus of research in our laboratory is on gene regulatory networks (GRNs) that control development, and the evolution of these networks. Most of our research is done on sea urchin embryos, which provide key experimental advantages. Among these are: an easy gene transfer technology, which makes this a system of choice for studying the genomic regulatory code; availability of embryonic material at all seasons of the year; an optically clear, easily handled embryo that is remarkably able to withstand micromanipulations, injections and blastomere recombination and disaggregation procedures; a very well understood and relatively simple embryonic process; and in-house egg-to-egg culture of the species we work with, *Strongylocentrotus purpuratus* (in a special culture system we have developed, located at Caltech's Kerckhoff Marine Laboratory). There is also a rich collection of arrayed cDNA and BAC libraries for sea urchins and a large EST database. The genome of *S. purpuratus* has now been sequenced at HGSC (Baylor) and annotated. A very extensive repertoire of effective molecular technologies for experimentation on sea urchin gene regulatory systems has evolved. The experimental model that we utilize for evolutionary GRN comparisons is another echinoderm, also of local provenance, the starfish *Asterina miniata*. The embryo of this animal proves to be as excellent a subject for gene regulation molecular biology as is that of the sea urchin.

We pursue an integrated, "vertical" mode of experimental analysis, in that our experiments are directed at all levels of biological organization, extending from the transcription factor-DNA interactions that control spatial and temporal expression of specific genes to the system-level analysis of large regulatory networks. It has become apparent that the only level of analysis from which explanations of major developmental phenomena directly emerge, is the system level represented by the sea urchin GRN.

The main research initiatives at the present time are as follows: ***i. Analysis of the gene regulatory network underlying endomesoderm specification in* S. purpuratus *embryos*:** At present about 50 genes have been linked into this GRN. The architecture of the network is emerging from an interdisciplinary approach in which computational analysis is applied to perturbation data obtained by gene expression knockouts and other methods, combined with experimental embryology. Regulatory and downstream genes required for skeletogenesis and for territorial specification have been isolated utilizing high-density arrayed cDNA libraries. A predictive model of the GRN has emerged which indicates the inputs and outputs of the *cis*-regulatory elements at its key nodes. Most of the individual projects reported below are contributing to understanding of this network. ***ii. Testing the cis-regulatory predictions of the GRN:*** The GRN was constructed essentially by integrating the results of a massive perturbation analysis of expression of individual genes with spatial and temporal expression data for these genes. It predicts the required specific regulatory inputs and outputs linking the genes within the GRN. These predictions are subject to direct experimental *cis*-regulatory test, and correction, if need be. We have now authenticated the predicted *cis*-regulatory inputs into genes at all but two or three of the key nodes of the current GRN. At these nodes are regulatory genes into which there are multiple regulatory inputs from genes elsewhere in the GRN, and multiple outputs to other genes in the GRN. For some regions of the GRN the analysis is approaching maturity, in that it extends convincingly from maternal inputs to cell-type differentiation. The best example is the GRN subregion determining skeletogenic

micromere specification. Overall, the results of these experiments are converting the GRN from a model proposition into a hard-wired map of the genomic control logic for this portion of development. ***iii. Completion of the repertoire of regulatory genes engaged in the endomesoderm GRN:*** We used the data emerging from the genome sequence project to identify and assemble computationally all gene sequences that encode transcription factors. The temporal patterns of expression of these genes have been determined, and for those genes sufficiently expressed in the embryo, the spatial patterns as well. A handful of regulatory genes have been identified in this manner that evidently play a role in endomesoderm specification, but that are not yet incorporated into the GRN as it now stands. These are now being linked into the GRN by perturbation and *cis*-regulatory analysis, but the analysis shows that the endomesoderm GRN is nearing completion (with respect just to regulatory genes, and just to the endomesoderm from 6 to about 30 hrs of development, i.e., early cleavage to the onset of gastrulation). ***Evolution, viewed as a process of change in GRN architecture:*** Starfish and sea urchins shared a last common ancestor about 500 million years ago. Thus, analysis of the GRN controlling endomesoderm specification events in the starfish embryo will reveal both the nature of functional change in the GRN, and conservation of features that are so essential that they have resisted alteration for half a billion years. Examples of both have now been documented. The underlying processes are of course change, or alternatively, conservation, of functional *cis*-regulatory features. To study this we are examining starfish/sea urchin GRN differences at the *cis*-regulatory level. In a separate, large-scale effort, we have nearly completed the isolation of BACs containing 12 genes the *cis*-regulatory elements of which are known in *S. purpuratus*, from genomic libraries of five different sea urchin species ranging from 15 to 250 million years since divergence from the lineage leading to *S. purpuratus*. These will afford the opportunity of studying by computational and experimental methods the process of *cis*-regulatory evolution, which is very poorly understood. ***v. Oral and aboral ectoderm GRNs:*** We have begun work on the GRNs that control oral and aboral ectoderm specification. This is an effort in collaboration with the lab of David R McClay at Duke to extend the same kind of causal, system level GRN analysis to the whole embryo. This means obtaining GRN architecture for oral ectoderm and aboral ectoderm. There is one additional early embryonic territory, the apical neurogenic region, which is being studied in other sea urchin laboratories. The aboral ectoderm generates a single cell type, but the oral ectoderm gives rise to several distinctly functioning domains: mouth, columnar "facial" epithelium, neurogenic ciliary band, and the ectodermal signaling stripes which determine the location of the skeletal rods. The approach is to obtain all the regulatory players expressed in oral and aboral ectoderm from the analysis of all genes encoding transcription factors predicted in the genomic sequence, and engage them in a provisional network by carrying out a matrix of perturbation experiments. The network will be anchored at the onset of the ectodermal specification process, of which the initial gene zygotically expressed on the oral side is *nodal*. The *cis*-regulatory module controlling early oral ectoderm expression of nodal is in our hands and its target sites should provide the link between the initial cytoplasmic anisotropy and zygotic gene expression. ***vi. Computational and experimental cis-regulatory model:*** To build a logic model of the information processing functions of a *cis*-regulatory element that relates the input kinetics (i.e., the temporal changes in relevant transcription factor levels) to its output, we have returned to the *cyIIIa* gene. The logic model of the regulatory system of this gene is being completed by additional mutational gene transfer experiments. ***vii. Various explorations by new methods and approaches:*** As always, we are trying to expand knowledge by use of novel technologies for analysis of the GRN and the genome. Current applications of new technology include increasingly widespread use of *in vitro* reengineered BAC recombinants, which we are supplying to the whole sea urchin field; use of these in first attempts to "redesign" the process of embryonic development, by introduction of altered regulatory subcircuits in novel spatial domains; and application of a newly developed quantitative imaging method for assessment of expression construct output. We have also developed a completely novel method for blocking expression of any gene whenever and wherever desired, though this has so far been tested only in sea urchin embryo skeletogenic cells. This method should allow us to determine the function of regulatory genes which have multiple activity phases in one of the later phases, in embryos which develop normally up to that point. ***viii. Computational approaches to regulatory gene network analysis:*** The GRN visualization software BioTapestry, developed by our collaborators Hamid Bolouri and Wm. Longabaugh at ISB, is now in wide use, and we are further expanding its capacities so that it will automatically generate allowed network architectures from machine readable time and space of expression data plus results of perturbation analysis. In addition, studies of network regulatory logic are being undertaken in collaboration with Professor Sorin Istrail of Brown University. Many additional computational genomics and other projects are summarized below. ***ix. Theory:*** A new conceptual approach to the evolutionary mechanisms which account for both change and conservation of animal body plans, based on modes of alteration of GRN architecture, was developed in collaboration with Doug Erwin of the Smithsonian Museum. An integrated treatment of genomic regulatory systems in development and evolution at both *cis*-regulatory and network levels is presented in a new treatise by the PI, "The Regulatory Genome" (Academic Press/Elsevier, 2006).

The Center for Computational Regulatory Genomics

CCRG Highlights

This is an exceptional year for the Sea Urchin Genome Resource. The first draft assembly of the purple sea urchin genome has been posted, the annotation of the predicted gene models has been completed to the first freeze and the publications reporting this work are being submitted for publication in the late summer or early fall. There will be an entire issue of *Developmental Biology* dedicated to the annotation of the sea urchin genome. *Science* has agreed to publish the genome presentation paper and several satellite papers as perspectives or reviews. It is not an exaggeration to say that this could not have been accomplished without the materials from the Sea Urchin Genome Resource. We provided to the Sea Urchin Genome Sequencing Consortium genomic DNA, BAC libraries, cDNA libraries, unpublished sequence data and scientific management. In addition we acted as liaison between volunteers from the sea urchin research community and the Baylor College of Medicine Human Genome Sequencing Center, the entity that performed the actual sequencing work.

Genomics Technology Facility

The operation of the Facility centers on the Genetix Arraying Robot, a large flatbed robotic arm with video camera used to produce bacterial macro-array libraries and filters. We currently maintain in -80°C freezers 27 different echinoderm libraries comprising a total of approximately 3 million arrayed clones. In addition to providing these materials to academic research groups, we also offer the opportunity for outside groups from Caltech and elsewhere to array and spot their own libraries. During the past year we have arrayed a total 262,820 colonies and printed a total of 129 macroarray filters

A variety of cell type-specific and tissue-specific cDNA libraries are especially useful for annotation processes because expressed sequences verify predicted gene models and additional tissue- and cell-specific genes are added with each new library. The cDNA library from primary mesenchyme cells was extensively used for this purpose. We have also made, arrayed and distributed two separate cDNA libraries made from the radial nerve tissue of adult sea urchins. A random collection of about 2,000 clones from these libraries was sequenced at Baylor. Similar numbers of clones from previously made gut, lantern, mesentery and testis were sequenced in this campaign.

We anticipate the need for additional genome resources to extend the recently obtained draft sequence. Gene clusters, long low-frequency interspersed repeats, and similar features are likely to require additional sequencing for proper inclusion in the draft genome sequence. To that end we have made a genomic BAC library using sperm from an animal that resulted from the self-cross of a hermaphroditic sea urchin from our colony. Theoretically this individual could have as many as 50% of the chromosomes completely homozygous. Of course recessive lethal mutations could reduce this number but nevertheless, a large fraction will be identical. We expect that this library will be especially useful for disentangling regions of problematic assembly since mosaic regions that would be otherwise difficult to assemble will be identical in all clones.

Research Center

The goal of the Center for Computational Regulatory Biology is to develop, refine and test computational approaches in genomics broadly and *cis*-regulatory analysis specifically. The primary focus for the latter is the elucidation of gene regulatory networks in development. The Center interacts with the wider research community in several ways: it provides open source software for use by academic research groups; it provides web-based servers for genomic analysis using software developed locally; and it maintains databases fundamental to the Sea Urchin Genome Project, an initiative that began in the Davidson laboratory and at the Genomics Technology Facility. The Facility provides to the Caltech and external scientific community upon request services and materials stemming from the macroarray libraries and arraying equipment that we maintain.

One aspect of the Center is the Sea Urchin Genome Resource which maintains information resources that are used widely in the sea urchin research community. We provide sequence information through the Sea Urchin Genome Project web site (http://sugp.caltech.edu/). With the advent of the web resources for annotation established at the Human Genome Sequencing Center, Baylor College of Medicine and the Sea Urchin Genome Resources at NCBI, we have not seen the need to expand our local databases. However, we have refined the cross-index between our library clones and sequences stored in public databases at NCBI. Since so many of our libraries were used for the sequencing project, and the library location for the clones was preserved in the sequence information, we can provide a searchable sequence database from which the user can obtain clone information and order the clone. This "clone by computer" method renders our arrayed libraries extremely useful and readily accessible for the working molecular biologist.

As in previous years our sequence analysis software and server continue to be productively used. 61 laboratories situated all over the US and Europe, and extending to Argentina maintain folders on our server. Since the last startup in February 2005, there have been almost 10,000 Seqcomp program job requests accomplished for a total of about 1250 CPU hours. The largest number of request has been for Blast jobs but they have only used about 834 CPU hours.

New Cluster Hardware and Configuration

We have replaced the set of 20 in-house assembled Pentium machines that previously comprised one-half of our Beowulf cluster with new Dell machines that achieve a significant increase in speed. To improve cluster deployment, maintenance, and management, we have adopted a proven software operating system and toolkit, NPACI Rocks, which is an open source, Linux-based system of software components that can be used to build, maintain, and operate the cluster. The toolkit was designed in November 2000 by the National Partnership for Advanced Computational Infrastructure led by the University of California, San Diego, and the San Diego Supercomputer Center.

313. Annotation of the draft sea urchin genome sequence

R. Andrew Cameron, Kevin Berney, Kris Khamvongsa

We continue to provide computational analyses in order to facilitate gene discovery and annotation of the genomic sequences as part of the Sea Urchin Genome Sequencing Consortium. We have modified our data pipeline to compare the assembled sequences to small candidate gene databases as part of the analysis strategies we have used during the manual annotation phase of the sequencing effort. Our curatorial work on gene families and functional groups has revealed several interesting aspects of the genes in the sea urchin genome. The spicule matrix protein genes function in the bio-mineralization process by which the clade-specific calcium carbonate stereom is secreted. Judging from comparisons to hemichordate and cephalochordate EST collections, these proteins are unique to echinoderms and are likely to have diverged from the larger C-type lectin gene family 540 million years ago. The most surprising expansion of a gene family in the sea urchin genome is the toll-like receptor family with ~220 members. These genes are involved in innate immunity and number only ten in humans, for example

Additionally, the predicted gene models derived from the sea urchin assembled genomic sequence were compared in several ways to the gene catalogs of other completed genomes. Thus, an overview of the sea urchin genetic toolkit was obtained and by extension the shared features of the gene catalogs that characterize other bilaterian animal groups was revealed. About one third of the most prevalent protein domains in the sea urchin gene models are not as abundant in the other genomes and thus constitute expansions that are specific at least to sea urchins if not to the complex of echinoderms and hemichordates that form the sister group to the chordate deuterostomes. A number of homologous groups of genes previously restricted to vertebrates have sea urchin representatives thus expanding the deuterostome complement. And obversely, the absence of representatives in the sea urchin confirms a number of chordate-specific inventions. Although the specific complement of genes in the sea urchin genome result largely from minor expansion and contractions of existing families already found in the common bilaterian "toolkit" of genes, several striking expansions shed light on how the sea urchin lives and develops.

314. Transcription factor utilization in larval development

Eve Helguero, R. Andrew Cameron

An analysis of the complement of transcription factors in the sea urchin genome reveals that about 80% are expressed during the embryonic period. This perhaps surprisingly large proportion is explained by the nature of gene regulatory networks, systems in which the interaction of transcription factors and the *cis*-regulatory modules that they bind is a stable but sensitive system that precisely controls development. The temporal utilization of the remaining 20% now becomes an interesting question. Previously, it would have been expected that a large proportion of transcription factors are first utilized in the larval period when the adult body plan is erected. We have begun to examine this issue by measuring the expression of these regulatory proteins in the larval period. We have established a method to dissect the primordium of the adult ventral surface away from other larval tissue and measure transcript number in RNA amplified from the tissue. Preliminary results on the 20% reveals that over half of them are expressed in the rudiment and these include many transcription factors known to be expressed during neural development in other systems.

315. Network Gene Annotation Project

Qiu Autumn Yuan, R. Andrew Cameron

The sequence and expression information that underlies the establishment of gene regulatory networks (GRN) is accumulating rapidly as high-throughput methods are adapted to these studies. We have inaugurated a database and website that is the used to collect these data for those genes identified in the course of various gene regulatory network efforts that are collaborations between the Davidson laboratory and other research groups studying sea urchin development. The database contains information on about 550 sea urchin network genes and is increasing daily. For each gene in the database the information includes fields with the annotations of the gene, its various sequence objects and expression data. This database has been newly implemented in PostgreSQL. The functions of the website and management of the database are scripted in a variety of languages: HTML, XML, CSS, PHP and Javascript. As these data are codified, they will be used to directly erect gene regulatory networks in a computational version of Biotapestry, our GRN viewer and editor.

316. The evolution of *cis*-regulatory module sequence in lower deuterostomes

R. Andrew Cameron, Elly Chow, Eve Helguero, Autumn Qiu Yuan, Ping Dong, Julie Hahn, Rena Schweizer

The DNA of *cis*-regulatory modules (CRM) known to be functional displays extensive sequence conservation in comparisons between genomes from modestly distant species**.** Patches of sequence several hundred base pairs in length within these modules are often seen to be 80-95% identical, while the flanking sequence cannot even be aligned. Comparisons of functionally characterized *cis*-regulatory modules from the *Strongylocentrotus purpuratus* genome, and the orthologous regulatory and flanking sequences from a BAC genome library of a congener, *Strongylocentrotus franciscanus,* reveal that single nucleotide substitutions and small indels occur freely at many positions within the regulatory modules of these two species, as they do without. However, large indels (>20 bp) are statistically almost absent within, though they are common in flanking intergenic or intronic sequence. With six cases analyzed, the indel suppression theory of *cis*-regulatory module sequence evolution is firmly established.

The studies of CRM evolution has been extended and broadened during the past year. We have added two new comparisons between *S. purpuratus* and *S. fransciscanus* to the analysis discussed above. Furthermore, we have expanded the panel of comparisons to be made. We now have partial results among five species and 10 genes. Recombinant BAC clones in which GFP is substituted for the gene-coding region while the surrounding genomic sequence is preserved have proved efficient constructs to test the regulatory function of large DNA segments. Constructs were obtained for six of the 10 genes in our panel from *Arbacia punctulata* (Ap), a sea urchin diverged from the reference species for about 150 MY. These are particularly interesting because only *delta,* a gene encoding a signaling ligand, was efficiently expressed. Thus, a new dimension of sequence evolution in our analysis panel is revealed.

317. Transcriptional control of the sea urchin *brachyury* gene

Elly Chow, R. Andrew Cameron

The *brachyury* gene is a participant in the endomesoderm specification pathway and the founding member of T-box family of transcription factors. Brachyury expression is localized to the vegetal plate and reaches a peak at about 24 hr postfertilization. By the 48 hr gastrula stage transcripts are present in the oral ectoderm and in the region of the blastopore. Expression then subsides and increases again during the larval stage. We have focused our attention on a construct that recapitulates the normal onset of expression in the vegetal plate. It contains 650 bp of the intron between exons 6, 7 and the previously identified *brachyury* basal promoter. From earlier perturbations and computational analysis we have discovered three classes of transcription binding sites likely to be active in this fragment: Gata, Tcf and Ets sites. When two of the four TCF sites are mutated, the expression is increasingly ectopic in the ectoderm at the expense of normal expression in the vegetal plate. These results are consistent with the notion that Tcf is an activator when bound to β-catenin that is localized to the nuclei of vegetal plate cells and an inhibitor through the co-factor, Groucho, in the ectoderm where nuclear β-catenin is absent. This hypothesis requires that additional activators are present in the ectoderm. We are investigating the possibility that members of the Ets family may fill this role since several canonical Ets factor-binding sites are in the sequence and two Ets factors Sp-Erf and Sp-ElfA are ubiquitously expressed at the right time. The discovery of a direct involvement for Tcf and β-catenin downstream of the feedback loop that activates vegetal plate specification is novel.

318. A genome-wide survey of sea urchin transcription factors

Meredith Howard Ashby, Stefan C. Materna, C. Titus Brown, R. Andrew Cameron

The sea urchin sequencing project has provided he opportunity to undertake a definitive survey of transcription factors involved in this organism's development. The goal is to identify new genes and characterize when and where during development these genes are expressed so that they may be incorporated into the current *Strongylocentrotus purpuratus* endomesodermal gene regulatory network (GRN), filling in any missing connections and rendering the model complete.

In all, 283 sea urchin transcription factors were identified, including members of the homeobox, sox, ets, nuclear receptor, forkhead bHLH and basic zipper families. Zinc fingers genes were not considered in this study. Phylogenetic trees were constructed for all the major gene families. The sea urchin in general has one ortholog for each set of paralogous transcription factor genes found in vertebrates, resulting in a regulatory toolkit between one-third and one-half the size of the vertebrate toolkit.

QPCR time courses from the fertilized egg stage through 48 hours of development were obtained for each gene. From this data, it is apparent that the vast majority, 75%, are used at least once during development. A separate study of the indicated that many of the remaining genes are used during the feeding larval stage and during elaboration of the adult body plan. Whole mount *in situ* hybridization (WMISH) for the more highly expressed genes shows that the newly uncovered genes are expressed in a wide variety of tissues, including the vegetal plate, primary mesenchyme cells, the gut, and both oral and aboral ectoderm. This information, along with perturbation analyses of currently known genes, will allow us to place these genes into the endomesodermal GRN being constructed in the lab, as well as present a starting point for the construction of an ectodermal GRN.

319. The C_2H_2 zinc-finger genes of *Strongylocentrotus purpuratus* and their expression in embryonic development

Stefan C. Materna, Meredith Howard Ashby, Rachel F. Gray

The C_2H_2 zinc-finger domain is one of the most abundant protein domains in metazoan genomes. With 22 amino acids it is small and named for two cysteine and histidine residues with which it coordinates the zinc atom. Genes carrying this domain are thought to have been extensively replicated in diverse animal clades. Many well-studied proteins that contain this domain are transcriptional regulators. As part of an attempt to delineate all transcription factors encoded in the *Strongylocentrotus purpuratus* genome, we identified the C_2H_2 zinc-finger genes indicated in the sequence, and examined their involvement in embryonic development. We found 377 zinc-finger genes in the sea urchin genome, about half the number found in mice or humans. This number, albeit preliminary due to redundancy issues, exceeds the number of all other, non-C_2H_2 transcription factors. We determined expression profile by quantitative PCR. Up to the end of gastrulation less than a third of these genes is expressed, and about 75% of the expressed genes are maternal; both parameters distinguish these from all other classes of regulatory genes as was found in complementary projects. Of all other transcription factor candidates about 80% are expressed by the end of gastrulation and only about one third of them is maternal.

Spatial expression patterns were determined by whole mount *in situ* hybridization for 44 genes transcribed at a sufficient level, and localized expression was observed in diverse embryonic tissues. These genes may execute important regulatory functions in development. However, the functional meaning of the majority of this large gene family remains undefined. Several examples from the literature point at additional roles for the C_2H_2 domain in RNA and protein binding. The functional flexibility together with their modular structure, which is thought to allow for rapid changes in binding site specificity, may be partly responsible for their high popularity in today's genomes.

320. *Trans*-specification of primary mesenchyme cells through genetic rewiring of the mesoderm specification network

Sager Damle

In the sea urchin *Strongylocentrotus purpuratus*, the identity and regulatory relationship of a number of transcription factors involved in endomesoderm development have been well characterized. However, the ultimate demonstration of intellectual control of the causal moving parts of a system is to reengineer it. The goal of the project is to determine whether the ectopic expression of the transcription factor *SpGcm* is sufficient to *trans*-specify primary mesenchyme cells (PMC) into a secondary mesenchyme cell (SMC) fate. This can be done by placing the *gcm* coding sequence under the control of a promoter that directs PMC-specific gene expression. The Davidson lab has developed a system whereby BAC-sized DNA fragments can be introduced into fertilized sea urchin eggs through microinjection and integrated into the genome as early as the two-cell stage. This system has been used here to probe the effects of creating novel connections between regulatory pathways.

SpGcm is thought to play two roles in development. Its early expression in all presumptive mesoderm suggests it is capable of setting up a mesodermal transcriptional state. This state gives cells a competency to respond to signals that specify various SMC or mesodermal cell lineages. Some evidence for this theory already exists. For example, embryos injected with antisense morpholino against *gcm* do not correctly express *gata c* gene in the oral domain of veg2 mesoderm (A. Ransick and J. Rast, unpublished data). The later role of Gcm in pigment cell specification is perhaps more difficult to characterize through morpholino-analysis. However, *in situ* hybridization shows late *gcm* expression exclusively in pigment cells.

The T-box transcription factor Tbrain is expressed in the large micromere descendants at swimming blastula stage. Its expression persists through gastrulation into PMCs. A recombinant BAC has been constructed whereby *gcm*-coding sequence is transcribed under the control of the *tbrain cis*-regulatory system. PMC cells expressing *gcm* have been shown to express a terminal differentiation gene, *pks,* which is normally transcribed only in pigment cells. *Gcm*-expressing PMCs also do not participate in skeletogenesis. Instead, they appear to migrate towards the aboral ectoderm, in a manner similar to their pigment cell relatives. The degree to which Gcm can both override the skeletogenic program of PMCs and specify either pigment cell or mesodermal cell fate has been assayed through whole mount *in situ* by measuring expression of other PMC and SMC-specific transcription factors and markers. Such factors include Gata-c, a mesoderm-specific transcription factor controlled by Gcm, Alx, a homeobox protein involved in PMC specification, and the forkhead factor Foxb.

Due to difficulties in resolving expression patterns using colorimetric WMISH, a fluorescent *in situ* protocol has been developed that will allow precise spatial measurements of gene transcription. In the context of this project, a two-color fluorescent *in situ* will be a valuable tool for measuring coexpression of induced GCM and the gene targets whose expression it regulates.

321. *cis*-Regulation of Spgcm

Andrew Ransick

A variety of experimental approaches are being employed to define the *cis*-regulatory architecture and critical '*trans*' inputs of *spgcm,* the sea urchin (*S. purp.*) orthlog of the *Drosophila* transcription factor *glial cells missing*. Four *cis*-regulatory modules of the *spgcm* gene have been identified (distributed across ~15 kilobases of sequence upstream of transcription start site) which when incorporated in a GFP expression construct, recapitulate both the early expression pattern of this gene in the

secondary mesenchyme domain at the mesenchyme blastula stage, and the late expression domain in pigment cells of post-gastrula stage embryos and larvae. Spatial expression of this GFP expression construct is severely disrupted by co-expression of dn-*Su(H)* mRNA, confirming that *spgcm* is a direct target of canonical N signaling mediated through Su(H) inputs. *cis*-Perturbation analysis of the "E module" (~350 bases located ~9 kilobases upstream of the transcription start site) using mutation of consensus Su(H) sites, identified a conserved motif paired-site and a lone site that function both to drive expression in SMC precursors which receive the Delta signal, and to repress expression in ectopic locations which lack this signal. While these Su(H) target sites provide the *cis*-regulatory architecture with the core of a N-signaling transcriptional response switch, both the *on* and *off* outputs from this module require additional inputs. The regulatory module controlling the late expression in pigment cells is the current focus. This so-called "G module" (spanning ~500 base stretch located ~4 kilobases upstream of the transcription start site) is composed of a clustered set of short conserved sequence elements, which includes a potential auto-regulatory site in the form of a consensus site match to the canonical GCM-binding site. Experiments are in progress to determine the specific sequence elements in G-module that account for the late expression pattern.

322. *cis*- and *trans*-regulatory analysis of *SpNodal* expression

Jongmin Nam, Yi Hsien Su

SpNodal is a member of TGFbeta gene family and is one of the earliest key components of oral-ectoderm gene regulatory network during sea urchin embryogenesis. The goal of this project is to understand the regulatory logic for initiating and maintaining the expression of *nodal* on the presumptive oral side of ectoderm. On the basis of earlier studies from other species, we reasoned that the regulatory logic for *nodal* expression is composed of the followings: 1) early and nodal independent input; 2) positive feedback input; and, 3) repressor inputs that limit *nodal* expression on the presumptive oral side of embryo. A series of perturbation studies and *cis*-regulatory module analyses suggested that all of the three predicted components indeed are parts of the regulatory logic for *nodal* expression. We are in the process of finding *cis*- and *trans*- elements of the logic.

323. Re-engineering the embryo by rewiring the regulatory network – endoderming the ectoderm

Smadar Ben Tabou de Leon

The instructions for cell specification and differentiation are primarily encoded in the regulatory regions of the genomic DNA that control the expression of transcription factors and signaling molecules. The inter-regulating factors create a network that is essentially the genomic program for development. Once the network is known it should be possible to re-engineer the embryo morphology by rewiring network components. This means that a network subcircuit that is believed to be sufficient for domain specification can be turned on in another domain and change its fate. If the domain fate changes according to the implanted subcircuit, it indicates that indeed this subcircuit is sufficient for domain specification. If the domain fate remains unaltered it is either that the implanted subcircuit is missing a necessary factor or that the endogenous fate was already established when the implanted subcircuit was turned on so the domain lost its plasticity. Therefore, rewiring the network is a unique tool that opens the way to deepen the understanding of network completeness and plasticity.

A central subcircuit in the sea urchin endoderm specification network is a positive feedback loop that is formed between the transcription factors *otx* and *gatae.* In order to check whether this positive feedback loop is sufficient for the endoderm specification we would turn it on in the ectoderm. For that end we prepared different bacterial artificial chromosome (BAC) constructs where *otx* and *gatae* cDNA were knocked-in into genes that are expressed in the ectoderm. The constructs will be injected into sea urchin fertilized eggs and induce expression of *otx* and *gatae* in the ectoderm. Different driver genes are used to turn on *otx* and *gatae* at different time points, early and late in development so we will be able to observe whether the plasticity of the ectoderm changes with time. We expect to see activation of endoderm regulatory genes in the ectoderm and possibly, formation of an ectopic gut demonstrating that the gene regulatory network architecture can be utilized to reengineer the embryo morphology.

324. A *cis*-regulatory analysis of *spgata-e*

Pei Yun Lee

SpGata e is the *S. purpuratus* ortholog to vertebrate Gata genes *4/5/6*. The expression of *SpGata e* is first detected in presumptive secondary mesenchyme cells (SMCs) during the hatching blastula stage. Its expression expands to include both future SMCs and endoderm in the mesenchyme blastula. In the gastrula, *SpGata e* is expressed at the tip of the archenteron and hindgut. By the end of embryogenesis, *SpGata e* is expressed in the midgut and coelomic pouches.

A 600 bp DNA sequence in the first intron is responsible for directing *SpGata e* expression in the vegetal plate from the onset of zygotic *SpGata e* expression in the 15 hr blastula. This module also maintains expression in mesoderm cells at the tip of the invaginating archenteron and endoderm cells until mid-gastrulation. When this module is deleted from a BAC containing the entire *SpGata e* gene and a GFP reporter, early expression was abolished while late expression was unaffected, demonstrating that it is required for the early expression of *SpGata e*. Further analysis showed that a separate 360 bp fragment in the first intron is responsible for directing endoderm expression in the gastrula and pluteus.

The endomesoderm gene network predicts that *SpGata e* is downstream of *SpOtx* and *SpNotch*. If the network prediction is correct, then functional binding sites must exist for these transcription factors in the *SpGata e* *cis*-regulatory region. A search in the sequence of the vegetal-specific *cis*-regulatory element identified three putative SpOtx-binding sites. Gel shift analysis has shown that the Otx transcription factor binds to the SpOtx-binding sites. Mutations of the sites abolish Otx binding. Furthermore, the vegetal-specific element also responds to perturbation of Notch signaling. When Notch signaling was perturbed in a reporter construct with mutated SpOtx-binding sites, GFP expression was abolished, verifying the presence of a *SpNotch* input into *SpGatae*. Closer examination of the module reveals four putative Suppressor of Hairless binding sites. Mutation of two of these leads to a marked decrease in the expression of GFP reporter.

325. Evolution of skeletogenesis gene regulatory network in echinoderms

Feng Gao

Although all Echinoderms (*Crinoidea, Holothuroidea, Echinoidea, Asteroidea, Ophiuroidea*) can build hard adult body parts (skeletal plates, tests, spines and so on), it is only in Echinoids and Ophiuroids that the embryo makes spicules that will be then discarded during later adult skeletogenesis. This begs the question: How did embryonic skeletogenesis – a novelty through Echinoderm evolution - arise?

The PMC (Primary Mesenchymal Cell) sub-network of sea urchin endomesodermal gene regulatory network (GRN) tells us the developmental regulatory program underlying the embryonic skeletogenesis in *Strongylocentrotus purpuratus* [1] [2]. This program involves dozens of specification and differentiation genes. Expression of all these genes was checked using WMISH of larval stage *S. purpuratus*. Preliminary data shows the same group of core genes used to make embryonic spicules, are also used at the larval stage to make adult skeleton. This finding indicates that part of the adult skeletogenic subcircuit was inserted into the embryonic PMC network during Echinoid evolution.

The orthologs of this GRN are currently being isolated from starfish *Asterina miniata*. Comparative expression analysis between Echinoidea and Asteroidea will be used to expand the understanding of the evolution of skeletogenic specification programs in this phylum.

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326. *cis*-Regulatory analysis of the sea urchin *delta* gene

Roger Revilla

The *delta* gene plays two different roles in the specification of the endomesoderm of the sea urchin embryo. Each one of these roles requires Delta to be localized in a specific territory of the embryo. It is first required in the micromeres to serve as a signal that is necessary to segregate the mesodermal and endodermal fates of the surrounding cells. It is later localized in the prospective SMCs, where it signals the endodermal cells and is required for gastrulation to occur. The goal of this project is to analyze the *cis* regulatory system that localizes the expression of Delta in the right place and the right time to serve its roles in the specification of the endomesoderm. It has already been shown that the early localization of Delta in the micromeres depends on activator(s) that are present ubiquitously, and a repressor that is present everywhere except in the micromeres. Comparison of genomic DNA sequences of *Strongylocentrotus purpuratus* containing *delta* gene with the orthologous region of *Lytechinus variegatus* genome has been used to identify conserved patches of sequence that might contain *cis*-regulatory elements. Two sequence elements have been identified that are able to recapitulate the two phases of expression of the *delta* gene. The element that recapitulates its early phase of expression has been shown to contain binding sites for activator(s) ubiquitously present and binding sites for the repressor that localizes *delta* in the micromeres. Future work will identify the sites in the DNA that bind these factors. Finally, we also hope to be able to identify the transcription factor that acts as a repressor of *delta* everywhere in the embryo except the micromeres, which has been suggested to play a key role in the installation of the skeletogenic program of gene expression.

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Postdoctoral Scholars: Doug Altshuler, Will Dickson, Mark Frye, Titus Neuman, Christian Poelma, Andrew Straw
Graduate Students: John Bender, Seth Budick, Gwyneth Card, Michael Reiser, Alice Robie, Rosalyn Sayaman, Jasper Simon
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Summary: Complex and intellectually challenging problems can be so commonplace that they escape our attention. The research in my lab focuses on one such everyday phenomenon - the motion of a fly through the air. While the buzz of fly wings is more likely to elicit a sense of annoyance than wonder, insect flight behavior links a series of fundamental processes within both the physical and biological sciences: neuronal signaling within brains, the dynamics of unsteady fluid flow, the structural mechanics of composite materials, and the behavior of complex nonlinear systems. The aim research in my lab is to elucidate the means by which flies accomplish their aerodynamic feats. A rigorous mechanistic description of flight requires an integration of biology, engineering, fluid mechanics, and control theory. The long-term goal, however, is not simply to understand the material basis of insect flight, but to develop its study into a model that can provide insight to the behavior and robustness of complex systems in general. The following projects in my lab, some well underway, others nascent and moving in the direction of a deeper understanding of the genetic control and brain of this insect, are helping move us in the direction of reverse engineering a fly.

327. GUF: Grand unified fly

Will Dickson

Flight, like all forms of locomotion, involves a complex interaction between an animal and its environment. Although neural circuits, muscles, and wings make up the central physical plant of an animal's motor system, flight behavior does not result from a simple set of feed-forward commands. For example, most of an insect's nervous system is dedicated to the sensory information that is generated as the animal moves through its environment. The insect's brain rapidly processes and fuses this rich information stream to create a motor code that can modify wing motion on a stroke-by-stroke basis. Sensory feedback is essential both for short-term stability as well as long-term guidance and navigation. What we view as behavior, such as a fly flitting across the room to land on the window, represents the output of a complex set of sensory-motor circuits that operates through the dynamics of muscles, skeleton, aerodynamics forces, and the environment. Although biologists have appreciated the central role of feedback in flight, conventional biological disciplines such as neurobiology or biomechanics are not endowed with the mathematical framework to deal with the feedback in a rigorous manner.

Fortunately, recent progress in insect aerodynamics has fostered new engineering approaches, such as the application of control theory, to animal flight. With this in mind we are developing a 3D dynamic model of the flight system of a flying insect, the fruit fly, *Drosophila melanogaster* (Figure 1). Flies provide a convenient scaffold for an integrated control model, because they have been subject to extensive investigations of aerodynamics, sensory processing, and motor control. The challenge is that certain features of our model are well constrained by detailed observation and experiment and easily formulated (e.g., wing and body aerodynamics), while other components are poorly understood and may be only roughly hewed in mathematical terms (e.g., neural circuitry and wing hinge mechanics). Despite this limitation, we feel that enough is known to warrant our attempt.

Our model is based on a framework that combines a simulation of insect flight dynamics, a model of insect sensory systems, and a model of the flight environment. Using this framework, control strategies can be evaluated by allowing the outputs of the sensory systems to modulate the forces produced by the wings and the results assessed in a quantitative manner. Such efforts are likely to help produce miniaturized, unmanned flying robots (robotic flies) in the future.

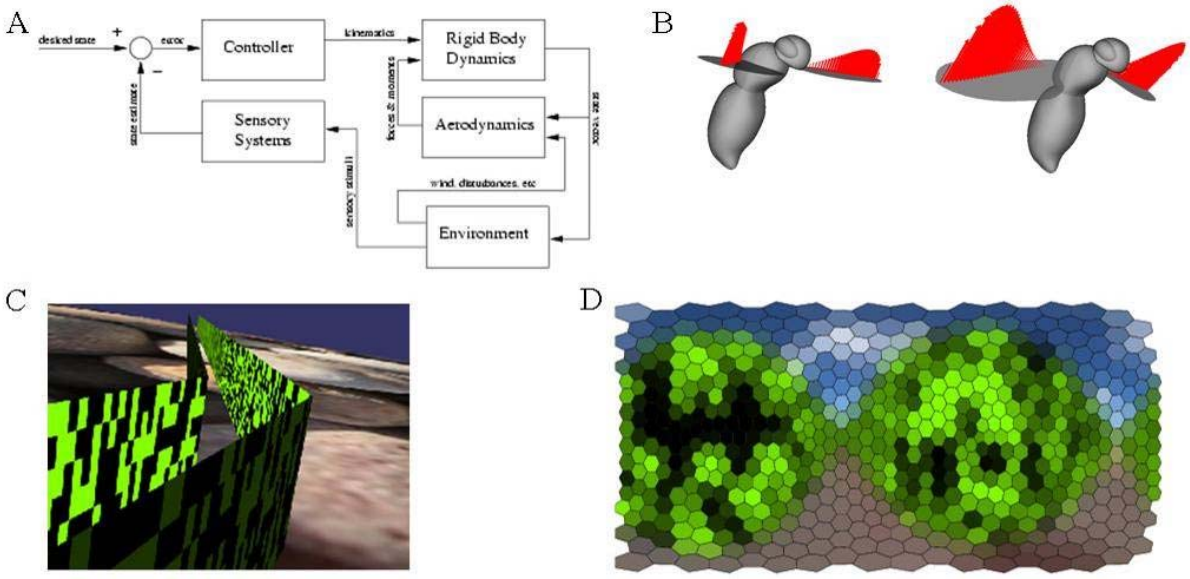


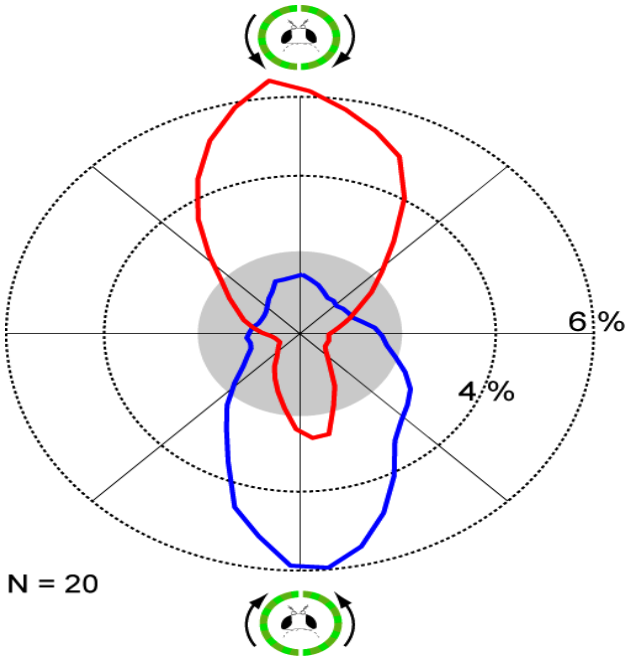
Figure 1. (A) Control block diagram for GUF. (B) Fly model rendered in 3D to show aerodynamic forces generated by each wing section. (C) Virtual environment for GUF simulation. (D) Fly view of the world.

328. The resolution of a paradox in the visually-mediated control of flight by *Drosophila*

Michael Reiser

Tethered *Drosophila* flying in a flight simulator will steer themselves by adjusting the difference between right and left wing stroke amplitudes; this difference is used to control the velocity of a rotating pattern. To simulate straight flight, flies were presented with a panoramic visual stimulus consisting of a pattern of stripes that diverges and an opposite pattern of stripes that contracts. In experiments, flies normally orient toward the contracting stripes. This original observation was perplexing given what you would expect a fly to see when flying through the world. One possible explanation for this apparent paradox is that the stimuli used are unnatural. For example, the expansion rate and contrast level are higher than would normally be encountered. The observed behavior may reflect an extreme response, such as an escape reflex. Our observations suggest a simple strategy for navigating in varied conditions—flies turn away from expanding objects at high visual speeds, thus avoiding collisions when they are imminent (since closer objects move faster). They balance optic flow when objects in their environment are farther away, thus flying straighter when it is safe to do so.

To further explore conditions in which the focus of contraction is fixated, the contrast level of the pattern was lowered (mean luminance preserved). To test the hypothesis that the focus of expansion may be tolerated if flies are undergoing directed flight, a high contrast stripe was embedded at the focus of expansion in half of the trials. This experimental condition essentially places two attractive stimuli in direct opposition, testing the relative attractiveness of both. When confronted with the compound stimuli, flies selectively orient towards the stripe at the focus of expansion. This result indicates that the expansion avoidance reflex is weakened when flies are fixating a visual object. This behavior suggests another sensible navigation strategy—orientation towards a prominent stationary object guarantees straight flight. Both behaviors suggest flight strategies that should enable straighter flight in the face of varying, complex visual conditions. Straight flight should serve to enhance the information coming from the fly's other sensory systems and thus, should enhance other visually-controlled behaviors. These predictions are currently being tested in a detailed computer simulation.



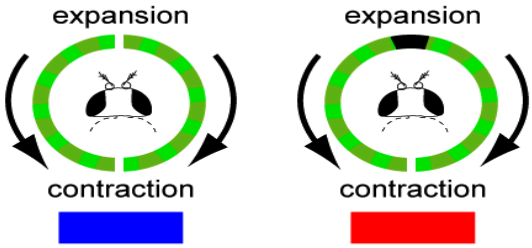


Figure 2: Tethered *Drosophila* tolerate expansion under modified conditions. These polar plots show histograms of the combined pattern positions during closed-loop flight. The grey filled circle corresponds to random orientation. Flies show a robust avoidance of the focus of expansion even under low contrast conditions (blue line); however the identical expansion condition is tolerated when a high contrast stripe is embedded at the focus of expansion (red line).

329. To land or not to land: Decision-making in *Drosophila*

Andrew Straw

The fruit fly is remarkably successful in implementing flying search strategies to find food and egg-laying sites in a wide range of external (wind, odor source, temperature) and internal (hunger, wing damage) conditions. We are investigating how the interplay of low-level sensory-motor reflexes might give rise to successful high-level outcomes, such as the decision of whether and how to land. Therefore, we developed a tracking system capable of resolving the position of a freely-flying fly over a large space in real-time while simultaneously capturing movies allowing detailed, post-experiment reconstruction of movements near potential landing sites. We first quantify the approach angle of a fly toward or away from a vertical post. Because this effect must be mediated visually, and because vision, among all sensory systems, is particularly well understood in the fly, we have constructed a realistic computer simulation of the visual system of *Drosophila.* Thus, we can reconstruct the view of the fly as it flew in the experimental arena, and we can make detailed predictions about the early stages of visual processing and use those to inform our understanding about how this information is used to decide whether to land. In this case, for example, the post is producing a strong expansion signal as the fly approaches.

Another interesting question about landing is how, precisely, does a fly control it's flapping to ensure its body follows a particular course? By utilizing the real-time nature of the tracking system, we can trigger very high frame rate (1000 or more frames per second) and high spatial resolution cameras to capture detailed wing and body kinematics during particular maneuvers. The pitch angle of the body is thought to be especially important in control of forward and vertical velocity, and digitizing such data will allow us to test models of the control system governing these variables.

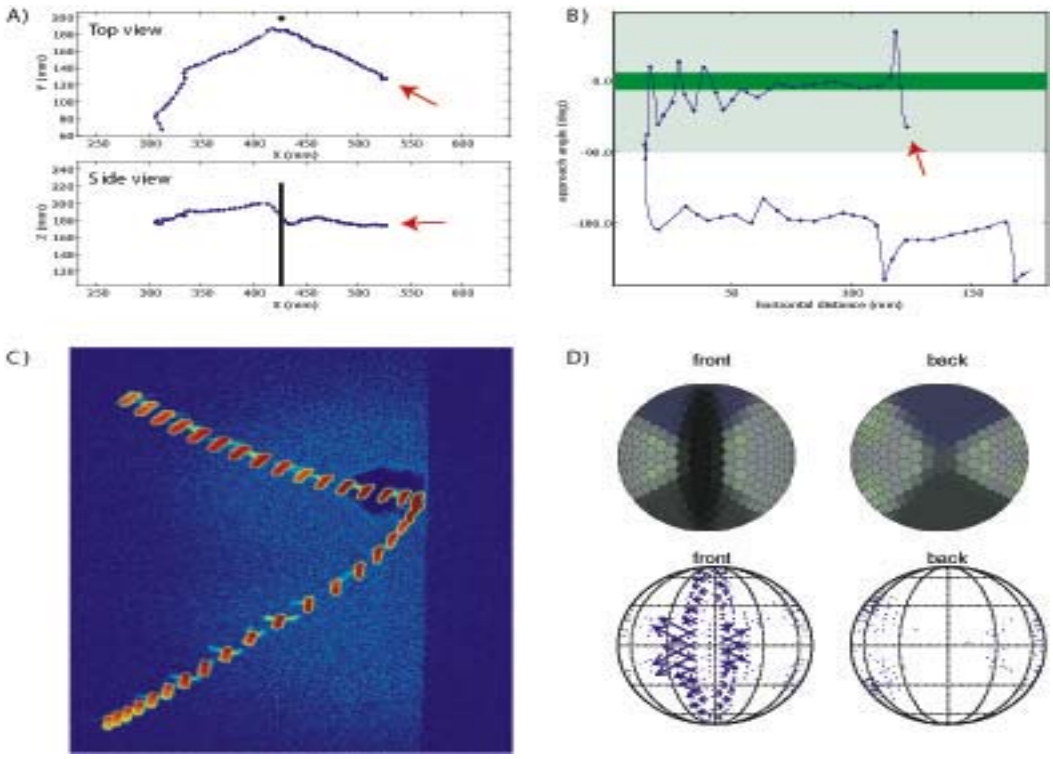


Figure 3. (A) Top and side views of a fly's trajectory in the vicinity of a vertical post. (B) During this period, the fly is either approaching directly or moving directly away from the post. Red arrows indicate the direction of travel. (C) A false-color montage of another trajectory in which the fly briefly contacts the post and leaves. (D) Reconstructed view of the image on a fly's retina as it approaches the post (top panels) and the simulated output of biologically realistic motion detectors viewing the scene.

330. Flight initiation performance in voluntary vs. escape takeoffs

Gwyneth Card

The initiation of flight is a complicated maneuver, requiring a high degree of coordination among appendages and wings. An animal must accelerate upwards while maintaining proper balance to remain stable. Different circumstances during takeoff may require varying performance requirements. For example, taking off to avoid a predator would require high speed but launching to track an odor plume of a potential food source or mate may require a smoother takeoff that allows an animal to follow its original orientation and heading. Behavioral studies of flight initiation of *Drosophila* suggest the fruit fly can perform two different types of takeoff, but these have never been compared in detail. One study showed flies taking off to follow enticing odors first raise their wings, then extend their middle legs while depressing their wings, thus coordinating leg movements with wing movements. In contrast, flies avoiding predators rapidly extends their middle legs and propel themselves off the ground without coordinating their legs and wings.

Using digital high-speed video (6000 frames per second), we have been able to capture and compare the performances of these two types of take-off in wild-type flies for the first time in detail (Figure 4). We found that flies accelerated primarily during the time they extended their legs for both voluntary and escape takeoffs but the period of leg extension was significantly shorter for escape jumps. As expected, the takeoff velocity was much greater for escape takeoffs than voluntary takeoffs, but this faster speed came with a cost. Escaping flies were far less steady, rotating rapidly and sometimes even turning upside down as they took off at extreme speeds. This lack of steadiness is not advantageous for controlled flight, but an obvious asset when avoiding predation.

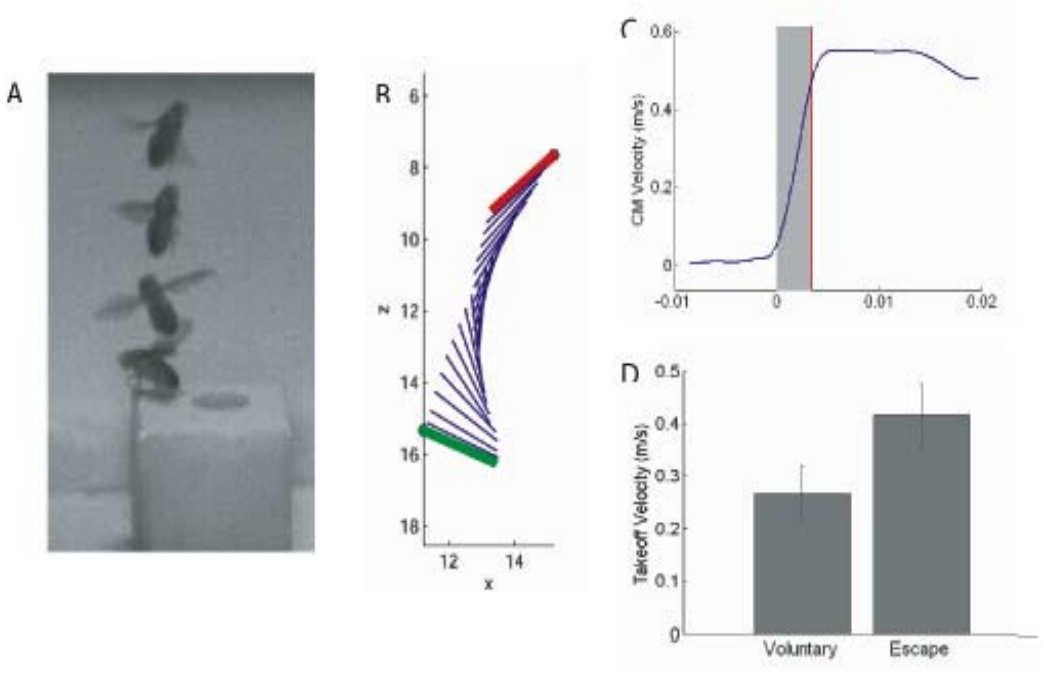


Figure 4. (A) Photo montage of an escape takeoff. (B) Digitized body axis of an escape jump, as viewed in the xz-plane. Green line indicates body axis position before takeoff (circle shows head end), and red line indicates body axis position in the final frame recorded. (C) Center of Mass velocity profile for the example escape takeoff shown in B. Time zero is when the legs started to extend. The shaded region shows the duration of leg extension. The red line indicates the moment when the legs left the ground. This is the time at which "Takeoff Velocity" was measured for all trials. (D) Pitch velocity for the same escape takeoff. The red lines mark the period after liftoff during which "steadiness" was measured.

331. Feeding and mating status govern dispersal from food resources in the *Drosophila*

Jasper M. Simon

It is crucial to an animal's survival to be able to decide whether to stay or disperse from an established resource, such as a supply of rotting food. The maturation of the fruit fly, *Drosophila melanogaster*, as a model system for neurogenetic research, allows us to revisit issues addressed in classic studies of dispersal carried out in the field and laboratory, with a new, more mechanistic perspective. Here we show results that suggest that feeding and mating status play a principle role in *D. melanogaster* dispersal. We developed an electronic vivarium – 'fly world' – a system of hardware and software to study the movement of flies within and between controlled sensory environments. We hope to use fly world to better resolve the contribution and interaction of the physiological, environmental, and genetic factors that together determine an animal's movement.

In one line of results (Figure 5), we found that if a chamber contained food, hungry flies stayed in that chamber longer than when no food was available, as expected. The effect was parametric: the more food, the longer they stayed. Next, we placed food behind a mesh surface, such that the flies could see and smell the food, but could not consume the food. Hungry flies given inaccessible food dispersed at the same elevated rate as hungry flies introduced to chambers without food. In contrast, sated flies dispersed at a similar slow rate irrespective of the amount or accessibility of food. Thus, visual and olfactory food cues are not enough to keep flies in one place. Flies must consume, or have contact with food, to inhibit their tendency to disperse.

In a second set of experiments, we examined the effects of mating history, sex, and sex ratio on dispersal. In the absence of food, we found that mated flies dispersed faster than virgin flies. This was true for both males and females. In the presence of food, males exhibited little dispersal irrespective of mating history, and previously mated females dispersed more slowly than virgin females. Curiously, the dispersal rate of virgin females was unaffected by the presence or absence of food. In general, mating looks to enhance the extremes of dispersal: When food is present mated flies stay longer, when food is absent mated flies disperse sooner. Finally, we placed males and females together in a chamber, and compared dispersal in this group to groups of only males or only females. From these results we suggest that the dispersal observed in mixed-sex populations is largely driven by males. Our data reveal that while inherent differences in dispersal exist between male and female flies, a requirement for food and not cues from food determines dispersal for both sexes.

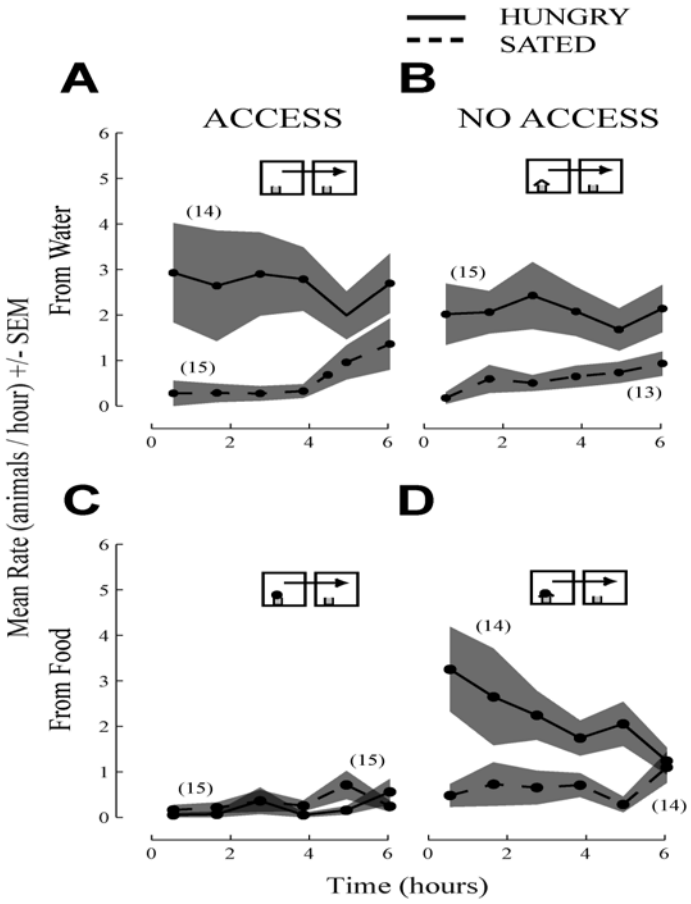


Figure 5. Hunger and absence of food inhibits dispersal. Hungry flies (solid) disperse from chambers in which food cues are present, but access to food is inhibited (D), at a rate comparable to dispersal from chambers in which food is not present (A,B). In contrast, sated flies (dashed) disperse at a similar slow rate irrespective of the presence and accessibility of food in the first habitat. We ran trials in all figure panels concurrently over several days. We monitored dispersal rates approximately every 10 seconds, but for clarity bin dispersal rates in 1-hour windows. We report samples sizes near mean dispersal rates and denote corresponding standard error of the means (SEM) in grey.

332. Control of body saccades in *Drosophila*

John Bender

Fruit flies exhibit a behavior known as "body saccades" in flight, which are changes in heading of about 90° in fewer than 100 milliseconds. These behaviors are a central way for flies to change direction and can be evoked by looming visual stimuli (such as an approaching flyswatter, or a large, growing rectangle inside a flight arena.) In order to elucidate the neural mechanisms underlying the generation and control of saccades, we developed a novel preparation in which flies retain some of the sensory feedback they lack under standard tethered conditions. We tethered a fly to a small, steel insect pin, which is held in the field between two magnets. The pin can rotate freely along its long axis. We are able to simultaneously record both the visual stimulus presented to the fly and the fly's turning responses.

We quantified how saccade dynamics were affected by manipulation of the fly's visual environment. We stimulated the fly with virtual looming objects. Stimulus properties had no predictive power for saccade dynamics, except for a slight effect of the position of the expansion relative to the fly's heading. However, the timing of saccades relative to the expansion did depend strongly on stimulus properties, in a manner suggesting that the saccade response is triggered by an angular threshold detector.

These experiments address the initiation of saccades, but we also tested the role of sensory feedback in saccade termination. First, we rotated the visual stimulus during spontaneous saccades. This rotation could be either in the same direction or the opposite direction from the saccade, but neither of these manipulations had any effect on saccade dynamics. This suggests that, while vision is important in evoking saccades, it does not play a major role in terminating them once begun.

Are saccades purely feed-forward behaviors, independent of sensory feedback? Flies possess an onboard gyroscope in the form of their halteres, modified hind wings sensitive to Coriolis forces. The haltere system is a likely candidate to provide feedback into the saccade motor program since it is highly sensitive to fast angular rotations, and can provided sensory feedback on a wingstroke-by-wingstroke basis, faster and more precisely than the visual system is capable of doing. We manipulated the feedback received by the fly from its halteres by adding mass to the endknobs of the halteres in some flies and ablating one haltere in others. We found that weighting the halteres significantly decreases the size of saccades, while ablating one haltere significantly increases saccade size. This demonstrates that the haltere system does play a significant role in saccade termination.

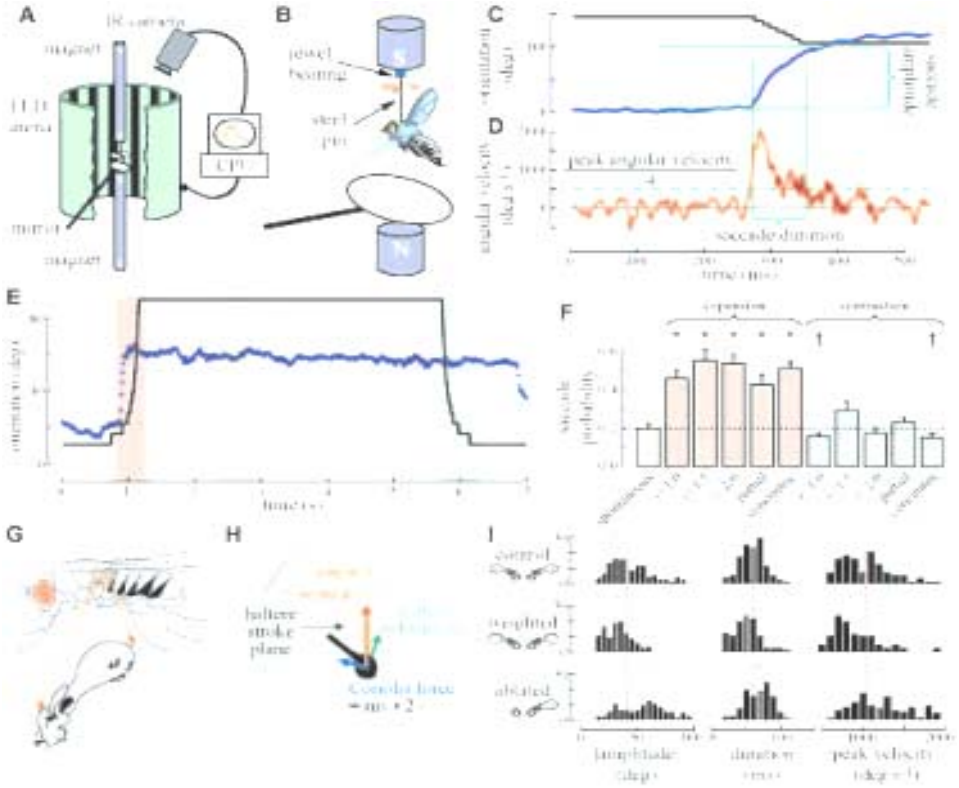


Figure 6. (B) Experimental apparatus. (C,D) Definition of saccade statistics. (E) Saccade stimulation paradigm. The half-angle subtended by an object approaching the fly follows the black trace as it expands until a virtual collision occurs, holds, then contracts as it moves away from the fly with the same time course. (F) Flies perform saccades in response to expanding objects (red bars). The specific stimulus type (varying velocities or geometries) did not affect the saccade probability. (G) Visual rotation during saccades did not affect saccade dynamics, so we tested whether haltere feedback was important. The halteres are the modified hindwings of dipteran flies, which respond to Coriolis forces proportional to the fly's angular velocity. (I) Altering the properties of haltere responses changes saccade dynamics.

333. Functional visual flight reflexes in *Drosophila*: Perch vs. predator

Gaby Maimon

Unmanned aerial vehicles require accurate environmental sensors, powerful flight actuators, and a robust control system to link them. While much is known about the design of sensors and actuators, the control systems required for these vehicles remain in their infancy. Our experiments seek to elucidate natural control systems within the flies that could then be replicated by engineers.

One simple higher-order neural control that flies perform is differentiating a perch, or sitting position, which they approach, from a predator, which they avoid. Our current experiments focus on a developing a quantitative way to characterize attraction and repulsion in flies. Future experiments will dissect the neural substrates of these behaviors.

Our experiments were performed on tethered, flying *Drosophila* placed in a flight arena that displays dynamic visual stimuli that can simulate predators (short stripes that appear as small, moving objects like wasps) or perches (long stripes that look like stable landscape features) (Figure 7). The experiments showed that flies consistently turned away from short stripes and turned toward long stripes. In addition, when placed in a closed-loop experiments in which the flies could control the movements of the stripes themselves, they tended to keep long stripes in front of them (fixation) and put short stripes behind them (anti-fixation), or let the short stripes spin unstably around the arena.

The data are consistent with flies using a simple visual heuristic – the ratio of length to width – to differentiate predator from perch. The two features – long stripe vs. short stripe – appear to be processed in separate visuomotor systems in the brain. These systems will be the subject of future analyses.

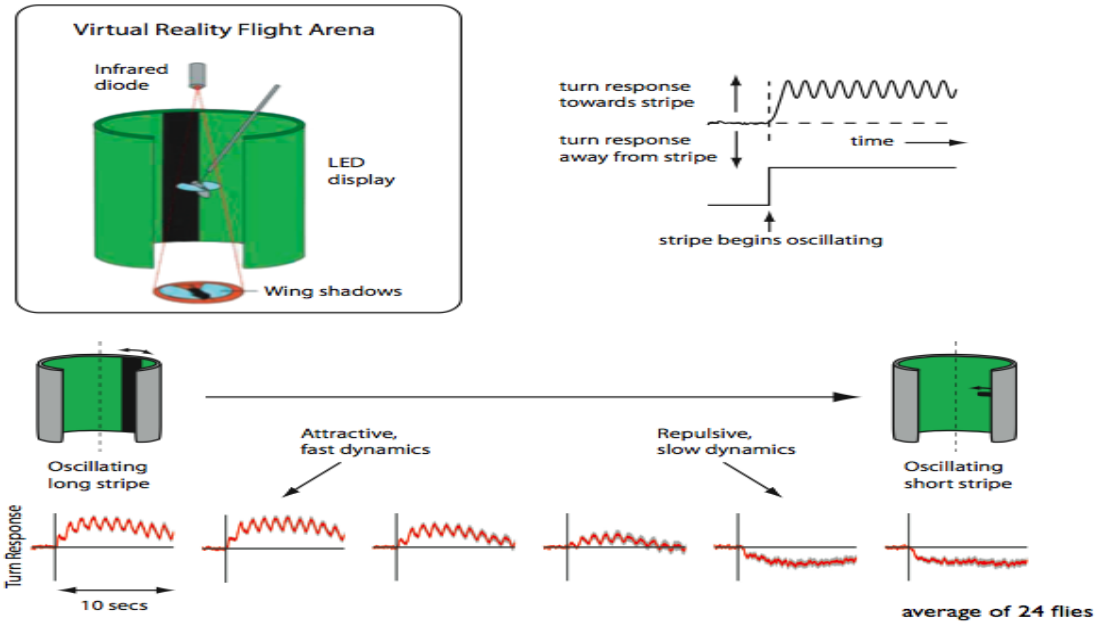


Figure 7. Turning responses to oscillating large ('perch') and small ('predator') stimuli. Flies turn towards the large stimuli, whereas they turn away from small oscillating stimuli.

334. Mechanisms of upwind odor tracking

Seth Budick

For a wide variety of insect species, olfaction is of central importance in locating both food and mates. The means by which flying insects search for attractive odor sources have been studied for decades, but research has tended to focus on just a few species of moths. It is thus, attractive to characterize odor source localization in the model species, *Drosophila melanogaster,* as this provides an important source of comparative data, allowing us to assess the generality of mechanisms of odor search. Our work has involved the study of behavioral responses both in free-flight and tethered-flight paradigms. In free flight studies using a low velocity wind tunnel, we have shown that *D. melanogaster* responds to contact with a narrow (1 cm diameter) plume of an attractive odorant by surging upwind within approximately 250 ms. This surge response is characterized by a shift from cross-wind to upwind heading, paired with a significant increase in airspeed. Flies subjected to continuous odor stimulation in a homogeneous odor plume, meanwhile, maintain extremely straight upwind trajectories while flying at increased velocities relative to a clean air control. Thus, while flies behave similarly to moths in surging upwind on odor contact, they do not share the common lepidopteran response of shifting to cross-wind casting flight in a homogenous odor plume. In additional studies, we have shown that when flies lose contact with a pulsed odor plume, they often initiate cross-wind casting flight as they attempt to recontact the plume, with casting occurring within approximately 300 ms of plume loss. Thus, *D. melanogaster* does not seem to require intermittent olfactory stimulation in order to maintain upwind flight.

We have recently subjected *D. melanogaster* to additional olfactory experiments in which flies were tethered while olfactory stimuli have been presented to them in the same wind tunnel used in our free-flight experiments (Figure 8). By analyzing the wing kinematics of flies exposed to continuous and pulsed odor plumes, we have shown that flies respond in a qualitatively similar way to both, with wing beat frequency increasing as a logarithmic function of the total duration of plume exposure. Flies responded more strongly to continuous plumes, suggesting a relatively simple algorithm whereby wing beat frequency is a simple function of total olfactory stimulation. Indeed, when we varied the duration between two brief odor pulses, the responses simply summed together. In the process, flies are able to track subsequent odor pulses via changes in their wing beat frequency at pulse frequencies up to 1.2Hz.

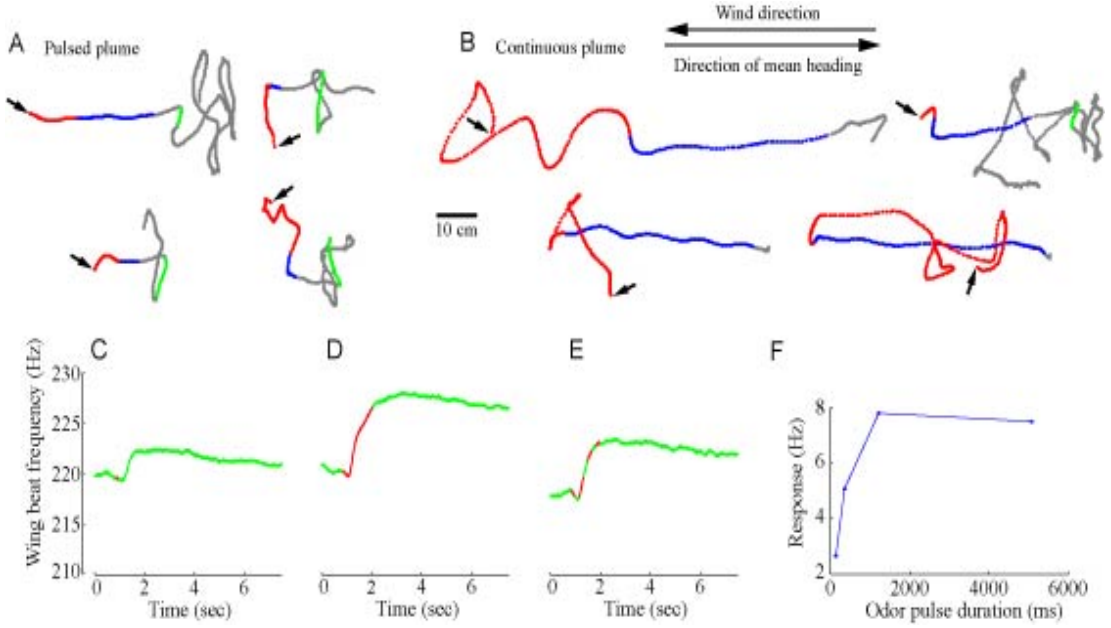


Figure 8. (A) Representative trajectories from pulsed banana odor illustrate flight prior to plume contact (red), within the plume (blue), and following plume loss due to truncation (gray). (B) In the continuous large diameter plume, casting rarely initiated within the plume. Arrows indicate the initiation of fly tracking. Behavioral responses are a function of total olfactory stimulation and are not increased in pulsed plumes. Flies were tethered and their wing beat frequencies were recorded under different odor presentation schedules. Odor was presented (red) in the form of a 140 ms pulse (C), a 1.3 sec pulse (D) four 190 ms pulses delivered over a 1.2 second interval (E) or, (not shown) a 360 ms pulse, a 5.4 sec pulse and 16 200 ms pulses delivered over a 5.2 sec interval. For continuous pulses, response magnitude increased as a logarithmic function of pulse duration with an apparent maximal response of approximately 8 Hz (F). Responses to continuous plumes were greater than those to pulsed plumes delivered over approximately the same duration.

335. Neuronal control of locomotor activity in the fruit fly

Allan M. Wong

When presented with visual or olfactory stimuli, the fruit fly changes its behavior. For instance, when exposed to an expanding pattern of visual stimuli, the fly will move away from an expanding object; when exposed to low concentrations of carbon dioxide the fly will move away from the gas.

In order to accomplish these behaviors, the fly must detect environmental changes, decide what to do, and coordinate its muscles to accomplish the behavior. We wish to understand how these steps are encoded in the nervous system of the fly. A good entry point is to understand how locomotor information is encoded by the descending interneurons. These neurons connect the brain with the thoracic ganglia (the equivalent of the spinal cord in vertebrates). All visually and olfactory-mediated behaviors must be transmitted through these neurons.

We performed preliminary experiments to identify these neurons using the light-activatable tracer GFP. We expressed this molecule in all neurons in the fly, light-activated a small spot in the neural pathway between the head and thorax and then examined the brain to reveal about 90 pairs of neurons.

We plan to record the activity of these descending interneurons while the fly is freely walking. In order to accomplish this, we built a walking arena modeled after Erich Buchner's design, where a tethered fly is walking on top of a floating ball. This allows for a preparation that simulates walking behavior while the fly is stationary, permitting functional imaging and electrophysiological recording with a two-photon microscope. We are also integrating the ball with the virtual flight arena hardware developed by Michael Reiser to present visual stimuli to the walking fly.

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Summary: Cells process information, interact with one another, and exhibit patterned development using circuits composed of interacting genes and proteins. Although the identities and many of the properties of these components are now known, it remains unclear how the circuits they compose function reliably within cells. In order to address these problems, we are applying experimental and theoretical techniques to key model systems in several different ways:

First, we construct synthetic genetic circuits and study their behavior in individual cells. These synthetic circuits are simpler counterparts to the complex circuits one finds in nature. This approach, often called "synthetic biology," allows one to analyze how various circuit designs might work, and begin to understand what is special about the specific circuit architectures observed in organisms. We have constructed circuits that exhibit oscillations and other dynamic phenomena, (e.g., Elowitz and Leibler, 2000). We have also used synthetic circuits to analyze the dynamics and variability of gene regulation at the single-cell level (e.g., Elowitz *et al.*, 2002, and Rosenfeld *et al.*, 2005). Current synthetic biology projects focus on regulation of two-component systems in bacteria, and higher-level developmental pattern formation in eukaryotic organisms.

Second, we analyze the dynamics of specific natural genetic circuits in order to understand basic principles of their operation. We have developed the ability to acquire and quantitatively analyze large time-lapse movie datasets. These movies allow tracking of circuit dynamics in individual cells as they grow and develop. By incorporating several distinguishable fluorescent protein reporter genes in these organisms, we can track several different circuit components simultaneously. The results constrain models of the corresponding circuits and provide insight into basic principles of their operation. A recent example of this approach is our work on regulation of genetic competence in *Bacillus subtilis* (see Suel *et al.*, 2006).

Third, we are pay particular attention to variability within cell populations. Variability is a basic feature of biological systems. Genetically identical cells appear to actively generate variability, even in homogeneous environmental conditions. We focus specifically on two complementary questions: How do cells use intrinsic "noise" (stochasticity) in their own components to make effectively random cell fate decisions? And how do they suppress noise in order to operate reliably despite of variability? Current projects are examining these issues in *Bacillus subtilis,* a prokaryote that exhibits relatively simple models of differentiation and development, as well as in the more complicated embryonic stem cell system.

Projects in the lab make extensive use of relatively simple mathematical models of genetic circuits. We are also developing software and tools to improve gene circuit construction and quantitative analysis of movie data.

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336. Transient and probabilistic differentiation in the competence response of *B. subtilis*

Gürol Süel, Rajan Kulkarni, *Jordi Ojalvo Garcia*

How do cells make random decisions about whether and when to differentiate? Can otherwise identical cells manage fluctuations, or noise, within their own components in order to effectively "roll the dice" when choosing cell fates? Recently, we have begun to use *Bacillus subtilis* as a model organism to address these questions. In *B. subtilis*, competence is a transient differentiated state in which cells can take up extracellular DNA. The decision to become competent is probabilistic and occurs in at most 10-20% of cells. Using time-lapse fluorescence microscopy movies, we analyzed the dynamics of the genetic circuit controlling competence at the single-cell level (Süel *et al*., 2006). Our results suggest that entry into competence and subsequent exit from it are controlled together by a core module of three genes that generate noise-excitable dynamics in a cell-autonomous fashion. (An excitable system, such as a neuron, is one in which a small perturbation can generate a well-defined response, such as an action potential.) This network makes use of a combination of positive and negative feedback loops to ensure that differentiation is transient. Additionally, we reengineered the competence circuit to access novel regimes of dynamic behavior, verifying a key prediction of the model. These results show that cells have evolved a dynamical mechanism which allows them to regulate the probability of competence much as a neural

system can control the firing rate of action potentials. More generally these results suggest that quantitative measurement of decision-making circuit dynamics in individual cells can reveal systems-level design principles.

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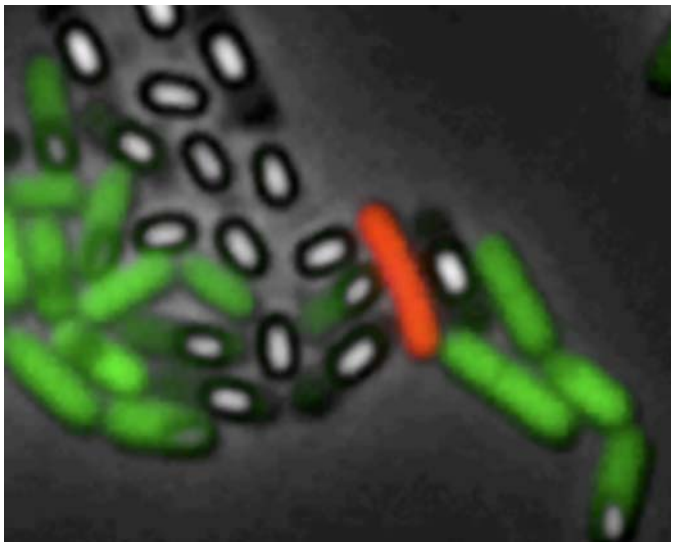

Figure 1: *B. subtilis* cells under nutrient-limited conditions. Here, some cells are sporulating (small phase bright (white) objects), while others grow vegetatively (labeled green), and one becomes genetically competent (labeled red). Genetic competence and sporulation represent two alternative stress response states that cells enter probabilistically.

337. Variability in sporulation initiation in *B. subtilis*

Joe Levine, Rajan Kulkarni

Under stressful conditions, *B. subtilis* cells respond by deciding whether, and when, to form robust spores that can survive harsh conditions and re-germinate later. Interestingly, even under conditions that favor this sporulation process, individual cells exhibit a large variability in the time it takes for them to initiate sporulation. This sporulation delay may function as a form of "bet-hedging" given the uncertainty of future environmental conditions that may improve (favoring vegetative growth) or may continue to deteriorate (favoring sporulation). The sporulation initiation circuitry has been well characterized using genetic and biochemical techniques, but it remains unclear how it functions to create long and variable delays in individual cells. In order to analyze the behavior of this circuitry at the single-cell level, we have constructed a variety of strains incorporating several different fluorescent reporter genes. Examining these strains with fluorescent time-lapse microscopy, we have observed variability in the expression of sporulation-specific genes which correlates with variability in the decision-making process. We are currently exploring how various dynamic mechanisms might generate this variability and looking for signatures of these mechanisms in gene expression patterns.

338. Phosphorelay dynamics

Shaunak Sen

Bacteria sense and react to environmental conditions using a variety of sensory systems. A dramatic example of how environment shapes bacterial behavior is in the starvation response of *B. subtilis*. When single cells of *B. subtilis* are in an environment with an abundant supply of nutrients, they grow and divide normally. However, when conditions are unfavorable, they make a decision to stop dividing and form spores.

The genetic circuit responsible for initiating this response consists of a signal transduction unit called a phosphorelay, embedded in a complicated set of positive and negative regulatory feedback loops. The goal of this study is to relate the architecture and function of different parts of this network. We have assembled a system that allows us to study the dynamics of the circuit in isolation from downstream effects. This work should provide a framework for establishing the sufficiency of a set of interactions in producing complex dynamics.

339. Developmental noise

Avigdor Eldar

Stochasticity, or "noise" can affect the individual steps of many developmental processes. Yet, developmental systems operate reliably most of the time. In order to understand how developmental systems suppress undesirable noise in their components, we are using the simple developmental process of sporulation in *B. subtilis.* Sporulation involves the asymmetric division of a single cell into a small (forespore) and large (mother cell) compartment. After division, the two cells initiate two separate, but tightly coordinated, programs of gene expression and morphological change. Eventually the forespore is engulfed in the mother cell which subsequently lyses. By constructing multiply-labeled fluorescent reporter strains of *B. subtilis* and imaging them using quantitative time-lapse microscopy, we are investigating the generation and propagation of noise in particular steps of this process. This effort should reveal general principles of noise resistance in development.

340. Directional selection modulates phenotypic fluctuations: On the interactions between selection and phenotypic noise

Chiraj Dalal

Many biological traits are quantitative: levels of gene expression, sizes of appendages, and abundances of cellular components can vary over a wide range. Mean values of such phenotypes are genetically encoded and subject to the forces of selection. Many such phenotypes also appear to be noisy: that is, genes specify a distribution of possible values for the trait, rather than a precise value. In some cases, it has been observed that the

variance of this distribution, like the mean, is under genetic control (Raser *et al.*, 2004). Recent work (Bar-Even *et al*., 2006, Newman *et al*., 2006) suggests that both means and variances of phenotypes are susceptible to selective pressures. However, for an individual in a population, only its (random) phenotypic value is under selection, not the underlying parameters of the distribution. Here we ask how positive directional selection affects variance in a simple quantitative trait. Analytically, we find that variance increases under strong selection and decreases under weak selection. Furthermore, we find that this result is not limited to variance, but also applies more generally to the noise of phenotypes. This observation opens up several interesting questions about how noise is generated and selected during evolution, which we are exploring through a combination of experimental and theoretical studies.

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341. Combinatorial *cis*-regulation of bacterial promoters

Robert Sidney Cox III

Combinatorial regulation of transcriptional initiation is a fundamental mechanism that cells use to integrate multiple inputs and control gene expression. We have constructed a synthetic library of combinatorial promoters, and developed a high-throughput assay for measuring the *cis*-regulatory input logic of these promoters in *E. coli.* A total of 48 pieces of synthetic duplex DNA fragments were randomly assembled by 5' overhang ligation and cloned into a luciferase reporter plasmid. Each promoter was then assayed in a combination of inducers to determine its logical response to each transcription factor. The library has an estimated diversity of 1,000-4,000 unique regulatory sequences, comprising a total of up to 400 kb of novel regulatory sequence. Each promoter is controlled by regulatory sequences for up to three of five transcription factors. The types of regulatory logic found in the library will provide insight into the regulatory mechanisms of natural combinatorial promoters, and how promoter architecture governs transcriptional logic.

342. A synthetic approach to pattern formation

David Sprinzak

Embryonic development frequently involves the spontaneous emergence of cell patterns within an initially equivalent field of cells. This process is required, for example, for the specification and development of neurons in the central nervous system in vertebrates. It has been proposed that this type of patterning happens by a mechanism known as 'lateral inhibition,' in which cells acquiring a desired cell fate prevent their neighbors from acquiring it. These patterning processes are executed through a combination of intracellular genetic circuits and direct cell-cell signaling, also called juxtacrine signaling. The canonical juxtacrine signaling throughout the eukaryotes is the Notch/Delta pathway, in which the membrane-bound ligand, Delta, on one cell interacts with the Notch receptor in neighboring cell.

Here we utilize a synthetic approach to study 'design principles' underlying this type of cellular patterning. We are constructing a synthetic genetic circuit that is based on the natural circuit of lateral inhibition. By utilizing the relative isolation of the synthetic system we can address some of the basic questions regarding the properties of the natural circuits controlling developmental differentiation: What are the design principles that govern these genetic circuits? What makes one circuit design perform better over others or be more robust than others? How do the properties of the signaling system affect the formation of the patterns?

To construct these synthetic lateral inhibition circuits, we are developing and characterizing several genetic components including both regulatory components and synthetic intercellular signaling systems. These components are integrated in mammalian cell culture via a novel genetic approach that enables a reproducible and quantitative investigation of the constructed circuits. The dynamics of these circuits are studied using automated time lapse microscopy of fluorescent protein reporters.

343. Analysis of gene circuitry underlying embryonic stem (ES) cell to trophectoderm stem (TS) cell conversion

Fred Tan

A fundamental question in development is how individual cells choose among possible cell fates. Embryonic stem cells now permit quantitative analysis of the dynamics of the gene circuits which control such decisions. Here we focus on specification of trophectoderm, which marks the first differentiation event of the mammalian embryo. Cells of the trophectoderm lineage form the placenta, an extraembryonic organ that interfaces maternal and fetal environments for nutrient and gas exchange. Molecular determinants involved in trophectoderm specification act early in embryogenesis (prior to the morula stage), but the existence of distinct cell types is not apparent until the formation of the blastocyst. Aside from the identification of a handful of genes absolutely required for lineage specification, the

underlying regulatory network governing trophectoderm fate specification remains to be characterized.

Recently, an analogous process of differentiation has been observed in mouse embryonic stem (ES) cells whereby transient ectopic expression of the gene *cdx2* is sufficient to generate a cell with morphological characteristics of trophectoderm stem (TS) cells. TS cells are a multipotent cell type that supports the development of the entire placental lineage. Cdx2 is thought to be a master regulator of the trophectoderm fate *in vivo* and its repression in ES cells is dependent on the pluripotency regulator Oct4. Although the role of Oct4 during *in vivo* trophectoderm specification is unknown, cross regulation between the two transcription factors may control fate specification in early blastomeres, despite epigenetic differences with embryonic stem cells (a population most similar to the embryonic epiblast).

The interesting pattern of cross-regulation between these two key developmental transcription factors represents an opportunity to analyze a cell fate decision at the single-cell level. To that end, we are building fluorescently labeled cell lines in which we will characterize gene regulatory dynamics between Cdx2 and Oct4 using mouse ES cells as a model system. We will connect the activities of Cdx2 and Oct4 to the emergence of TS characteristics and the loss of pluripotent markers to better compare *in vitro* and *in vivo* differentiation programs. We will quantitatively analyze expression dynamics of these key transcription factors and their target genes and use this data to develop a model of cell fate specification. In this way, we hope to understand how interactions among key transcription factors permit cells to exist as discrete lineage types, and how Cdx2 expression alone is coupled to a developmental program that reinforces differences between cells to initiate the formation of a distinct cell lineage.

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Summary: Our laboratory has dedicated itself to an integrative approach to defining the cell and molecular basis of embryonic patterning, in which *in vivo* imaging tools play a central role. The explosion of data from molecular approaches and the dramatic progress from *in vitro* culture assays have resulted in a rich set of proposals for the mechanisms that underlie developmental pattering. Systems biology provides a means to organize this wealth of data, but requires some means to test the proposed linkages. Our goal is to test these proposed mechanisms in the intact embryo, with the hope of moving forward to an understanding of which of the potential mechanisms operate in the natural biological context. There are many challenges to such tests, including the tagging of cells or molecules so that they can be followed in the intact system, the visualization of the tagged structures, and the interpretation of the time-varying events these images represent. Solutions to these challenges require the coordinated efforts of researchers spanning the life and physical sciences.

In the past year we have made significant advances in understanding the formation of the cardiovascular system in the zebrafish embryo. A major challenge in studying the development of the heart has been that key developmental events take place while the heart is beating twice a second. To answer this challenge, we have helped to refine a new microscope, now available commercially, with the ability to collect confocal laser scanning images at rates of 100's of frames per second. This permits the direct imaging of events within a normal embryonic heart as it is undergoing its dramatic morphogenesis, forming chambers and valves. New image processing tools allow us to assemble time-series data from many optical sections into a four-dimensional rendering of the moving cardiac cells together with detailed maps of the blood flow. New image analysis techniques permit quantitative analyses of these motions, and are permitting tests, in collaboration with the Gharib Lab (Bioengineering, Caltech), of the roles fluid flows and forces play in the shaping of heart chambers and valves.

In parallel with the refinement of new imaging tools, we have been creating new a more efficient means for creating embryos with genetically-encoded fluorescent tags. Through random insertion into the genome, this approach permits the creation of functional fusions between a host protein and a fluorescent protein, permitting cells and developmental questions to be posed in normally developing embryos. The creation of these new lines is dramatically more efficient than previous approaches, making it possible for even modest sized facilities to embark on large-scale screens. This increased efficiency of creating marked strains requires a parallel increase in imaging technologies, and the refinement of *in toto* image acquisition and analysis tools answers this challenge.

There have been dramatic advances in other areas as well, ranging from the refinement of new sensor technologies with researchers in the Kavli Nanoscience Institute, to improved tools for acquiring information from MRI images. Our combination of modern and classical technologies continues to offer surprises, including the discovery of a novel sensory pathway in the mammalian olfactory system.

344. Towards a digital fish: *in toto* imaging of zebrafish development

Sean Megason, Scott E. Fraser

A representative image from an *in toto* image set of the developing inner ear of the zebrafish. Nuclei are marked green using Histone2B-EGFP and cell membranes are marked red using a membrane-localized mCherry. We are trying to determine the complete lineage of the inner ear.

I am interested in studying zebrafish development using *in toto* imaging, a novel technology I am developing. The goal of *in toto* imaging is to digitize *in vivo* data in a quantitative and high-resolution manner, which is essential for the emerging field of systems biology. *In toto* imaging, in which nearly every cell in a developing embryo or tissue can be tracked through space and time, may become a standard technique for small transparent embryos such as zebrafish, *C. elegans*, and early-stage chick and mouse embryos. There are several technical challenges that must be met in order to permit *in toto* imaging. Firstly, embryos must be labeled in such a way to allow all of the cells in the region of interest to be individually distinguishable so they can be segmented. I have developed a labeling method using Histone2B-EGFP and membrane localized mCherry to label all the nuclei green and cell membranes red. This labeling technique allows each cell in a tissue to be segmented and reveals cellular morphology very nicely. The next technical challenge that must be met for *in toto* imaging, is that embryos must be imaged at sufficient spatial and temporal resolution to allow every cell to be tracked during development without damaging the embryo. We have developed mounting, confocal imaging, and data storage techniques that make it possible to continuously image a zebrafish embryo at very high spatial and temporal resolution for 48 hours and to successfully archive the gigabytes of images. The final and most difficult challenge for *in toto* imaging is to actually segment and track the cells in the image set. I have developed a software package called *GoFigure* for this purpose. *GoFigure* can automatically segment cells in image sets and connect the segmented cells to form cell tracks and cell lineages. I am first applying *in toto* imaging for determining the full lineage of the inner ear of zebrafish, but I would like to extend these methods to the whole embryo. We are also developing methods to fluorescently mark a wide range of biological data on a genome-wide scale such that it can be digitized with *in toto* imaging.

345. Four-dimensional image reconstruction and analysis for quantitative study of the embryonic heart

Michael Liebling, Arian S. Farouhar, Morteza Gharib[*], Scott E. Fraser, Mary E. Dickinson[**]

The temporal alignment of nongated slice-sequences acquired at different axial positions in the living embryonic zebrafish heart permits the reconstruction of dynamic, three-dimensional data (four-dimensional imaging). This approach overcomes the current acquisition-speed limitation of confocal microscopes for real-time 3D imaging of fast processes. We developed synchronization methods to perform either uniform or nonuniform temporal registration. When data is uniformly scaled in time and aligned, slight variations in the heart rhythm that occur within a heartbeat are not compensated. Since this imposes constraints on the admissible data quality, we derived a nonuniform registration procedure based on the minimization of the absolute value of the intensity difference between adjacent slice-sequence pairs. The method compensates for temporal intra-sample variations and allows the processing of a wider range of data; in particular, it permits the reconstruction of datasets even when some slices contain aperiodicities. The 4D reconstructions, based on data acquired in living, fluorescent zebrafish embryos and combined with quantitative analysis approaches, have enabled the generation of heart function models, providing information about wall motion, muscle contraction, cushion and valve motion, and blood flow, depending on which transgenic fluorescent protein marker line was used.

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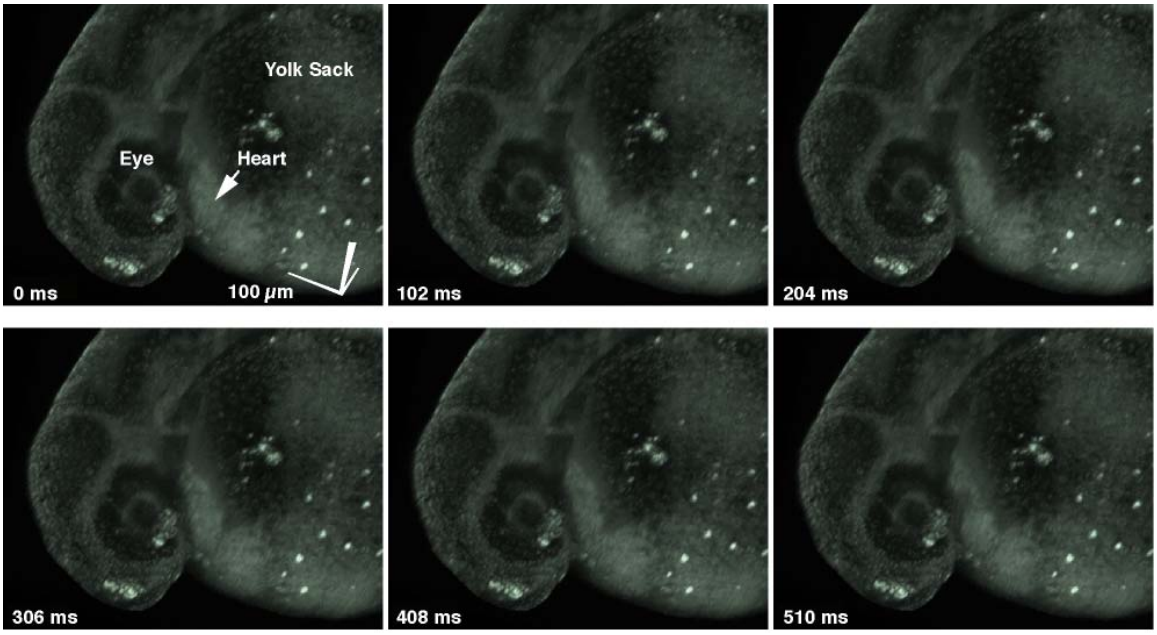

346. Investigating the role of the Grueneberg ganglion in mammalian olfaction

David S. Koos, Scott E. Fraser

The Grueneberg ganglion is a small nerve located in the lining of the far rostral rodent nasal vestibule. Historically, this nerve was thought to be a component of the terminal nerve, a non-sensory neuroendocrine nerve that does not innervate the first relay station of the primary olfactory pathway, the olfactory bulb. After noting that the Grueneberg ganglion expresses the pan-olfactory marker Olfactory Marker Protein (OMP), we suspected that this ganglion might instead be part of the olfactory pathway. Through the use of axon tract tracing techniques we have demonstrated that the Grueneberg ganglion projects its axons to a little known specialized sub-domain of the olfactory bulb, referred to as the olfactory necklace glomeruli. The expression of OMP combined with its direct wiring to the olfactory bulb suggests that the Gruenberg ganglion is a previously unrecognized component of the primary olfactory pathway. Our current work is focused on elucidating the axonal behaviors and molecular coding that underlie the connectivity of this ganglion with the necklace glomeruli. These experiments will provide important insight into the molecular nature and potential chemosensory function of this unusual olfactory nerve.

Publication

Koos, D.S. and Fraser S.E. (2005) The Grueneberg ganglion projects to the olfactory bulb. *NeuroReport* **16**:1929-1932.

347. Epithelial morphogenesis during heart tube formation

Le A. Trinh, Sean Megason, Scott E. Fraser

Epithelial cells provide the structural basis for organ development; therefore, understanding the molecular and cellular biology of epithelial formation will provide fundamental insights into the principles underlying organogenesis. To this end, we are using the embryonic heart tube as a model to study the dynamic cellular interactions of epithelial cells during organ formation. To assess epithelial cell behavior, we are performing four-dimensional imaging of transgenic zebrafish lines that mark the myocardial precursors, the epithelial cells of the developing heart tube. Using a green fluorescent protein fused to a membrane localization of signal (membraneGFP), we detect extensive filapodia formation between the cells of the myocardial epithelia as they migrate to the midline. Furthermore, we find that myocardial precursors delaminate from the bilateral epithelial sheets. We are currently testing the hypothesis that these delaminating cells undergo a differentiation switch to become endocardial cells. The availability of mutations previously identified in forward genetic screens affecting epithelial formation provides a genetic framework into which these cell behaviors can be placed. The analyses of wild-type and mutant cell behaviors during heart tube formation should further facilitate our understanding of the complex cellular interactions during cardiac development as well as the mechanisms underlying epithelial morphogenesis.

348. Genetic and epigenetic of bilateral symmetry in vertebrate embryo

Julien Vermot, Scott E. Fraser

The LR asymmetric positioning of internal organs is in contrast with the bilateral symmetry of the musculoskeletal and dermal outer layers of the body wall in most, if not all, vertebrates. The bilateral symmetry arises from the synchronized segmentation of the paraxial mesoderm on both sides of the neural tube into aligned pairs of somites along the anterior–posterior (AP) axis. The LR axis arises from a complex cascade of epigenetic and genetic mechanisms that ultimately results in the directional transfer of laterality information to the regions adjacent to the embryonic node by the late gastrulation and early somitic stages. Recent studies demonstrated that the Notch signaling pathway is required for the determination of the LR axis and the symmetry of somite formation, while retinoic acid (RA) protects the paraxial mesoderm from laterality determination to maintain bilateral symmetry. On the other hand, cilia rotating in the embryonic node act as an epigenetic mechanism responsible for breaking early embryonic symmetry and induce left-right axis specification.

In order to better understand the mechanisms maintaining the axis of symmetry, we designed tools to induce asymmetric growth and disrupt the bilateral symmetry of somite formation in chick (*Gallus gallus*). Our result shows that the synchronization of somite formation is actively controlled between the two embryonic sides. We are currently identifying the signals involved in this process using genetic tools available in fish (*Danio rerio*). Furthermore, we study cilia rotation by using high speed imaging in order to understand their role in instructing the left-right axis and the axis of symmetry. For that purpose, we are currently developing tools that will help us to understand the hydrodynamics and the flow generated by cilia in fish.

349. Assessing mobility differences of cytoplasmic GFP in zebrafish neuronal growth cones with multi-photon FRAP

Rajan P. Kulkarni, Scott E. Fraser

Navigating growth cones need to integrate, process and respond to guidance signals, requiring dynamic information transfer within and between different compartments. Studies have shown that faced with different navigation challenges, growth cones display dynamic changes in growth kinetics and morphologies. However, it remains unknown if these are paralleled by differences in their internal molecular dynamics. To examine if there are protein mobility differences during guidance, we developed multi-photon fluorescence recovery after photobleaching (FRAP) methods to determine molecular diffusion rates in pathfinding growth cones *in vivo*. Actively navigating growth cones (leaders) have consistently longer recovery times compared to ones that are fasciculated and less actively navigating (followers). Pharmacological perturbations of the cytoskeleton point to actin as the primary modulator of diffusion in differently behaving growth cones. This approach provides a powerful means to quantify mobility of specific proteins in neurons *in vivo* and reveals that diffusion is important during axon navigation.

350. Hydrodynamics of the zebrafish Kupffer vesicle

Julien Vermot, David Wu, Scott E. Fraser

All vertebrates have a clearly defined anterior/posterior and dorsal/ventral axis. Less noticeable is the left-right axis that exists primarily in the internal organs. The present hypothesis for left-right asymmetry breaking in zebrafish involves transport of signaling packets from one side of the Kupffer vesicle to the other, in spite of continual circulation in the spheroid vesicle. The Kupffer vesicle is a ~70 μm cavity that arises near the tail of the embryo at around 11 hours post fertilization. The circulation inside the vesicle is generated by the ciliated lining, and the mechanism by which cilia create flow is also controversial. By modeling the cilia-generated hydrodynamics and by measuring the *in vivo* flow field inside the Kupffer vesicle with optical tweezers, we hope to elucidate the mechanism by which circulation is generated, and illuminate the physical aspects behind left-right asymmetry breaking.

351. Non-equilibrium statistical mechanics of two-state systems

David Wu, Scott E. Fraser

In equilibrium statistical mechanics, the Gibbs algorithm for calculating probability distributions is a workhorse that consists of maximizing the entropy under certain constraints on the ensemble, such as a constant particle number, energy, etc. However, there is no reason why this concept cannot be extended to non-equilibrium systems. In equilibrium, maximizing the entropy is nothing more than a statement that the underlying degeneracy of microstates should be maximal; equivalently, there are relatively few high-energy states. In non-equilibrium, instead of degeneracy of microstates, there are degeneracies of microtrajectories. We seek to test this hypothesis experimentally. Using scanning optical tweezers, we generate microjectories by forming a W-shaped potential on which a colloidal particle may diffuse: this can be thought of as a two-state system, wherein the colloid takes different trajectories along the curvature of the W-potential, each state being defined as one half of the potential. Here, using certain constraints such as the relative time spent in one state versus another and the switching rate between states, we maximize the trajectory entropy that should give us the probability distribution of a non-equilibrium system.

352. Magnetic bead localization as a tool for interference with zebrafish development

Luca Caneparo, Scott E. Fraser

During animal development a single cell is able to form different tissues and cell types. One of the mechanisms, used several times during animal development, to create diversity is the asymmetric cell distribution of developmental determinants. The differential localization of signals during development is a mechanism repeatedly used among animals. For instance localization of β-catenin soon after fertilization is required to correctly position the axis in amphibian and fish embryos; later in development a similar strategy is used by neural precursors to give rise to differential cell population in mouse cerebral cortex. The unequal distribution of determinants it plays a key role during embryogenesis and is required for the correct development in different species.

In order to understand the mechanisms of differential localization and to try to selectively perturb such events we test the possibility to use magnetic beads to modify cell behavior at different time points during zebrafish development. Zebrafish embryos injected at one cell stage with fluorescent magnetic beads (1 μm in diameter) coated with carboxyl group show no preferential localization or toxicity at 5 days post fertilization (5 dpf) once injected at one cell-stage. The carboxyl group present at the surface gives the opportunity to link proteins to the beads, so as a test experiment we coupled Cherry proteins to the magnetic beads with similar results once injecting the modified beads at one cell stage.

Soon after injection we applied a constant magnetic field in proximity to the embryos and we were able to localize at the cell cortex the majority of the beads. Reorienting the embryos with respect to the magnet allowed us to reposition the magnetic beads. This validates the possibility of functionalizing beads with developmental determinants and localizing the signals using an external magnetic field.

353. *In vivo* 4D magnetic resonance microscopy of neurulation and somitogenesis in amphibian embryos

J. Michael Tyszka, Yun Kee, Russell E. Jacobs, Marianne Bronner Fraser, Scott E. Fraser*

Magnetic resonance microscopy offers a unique window on deep tissue movements in optically opaque embryos. The aim of this project is to develop MR microscopy with sufficient spatiotemporal resolution to explore key developmental processes during neurulation and somitogenesis in established amphibian embryonic models. The *Xenopus laevis* (African Clawed Frog) embryo is a classical vertebrate model of early embryonic development. Recent studies of vertebrate embryos have demonstrated the potential of MR microscopy in developmental biology, particularly in gastrula-stage frog embryos (1,2). Optical imaging techniques such as confocal laser-scanning microscopy are essential in the biological imaging field but are limited by the optical opacity of the organism. MRM may have a valuable role to play in this area since it is unhindered by optical opacity. We describe here initial experiences with high field MR microscopy studying neurulation and somitogenesis in single and multiple frog embryos. Wild-type *Xenopus laevis* oocytes were harvested, fertilized and incubated at room temperature (18-20°C) prior to imaging. A T_1 contrast agent was microinjected into either (a) both cells of the two cell-stage embryo or (b) the blastocoel fluid space of the blastula (stage 8-9) reducing T_1 to less than 200 ms. Volumetric spin echo MRM data were acquired using an 11.7T 89 mm vertical bore microscope (Bruker Biospin, Billerica, MA). Single embryos were mounted within a 1.8 mm id water-susceptibility matched tube that was subsequently sealed with dental wax to minimize buffer evaporation. Spatiotemporal resolution for a nominal SNR of 5-10 was 10 to 20 minutes/volume with 19 μm isotropic sampling for single embryos. Volumetric time-courses of normally developing *Xenopus laevis* embryos were acquired over the complete course of neurulation and early tailbud stages. Gadoteridol did not cross the embryonic cell membranes, remaining in the labeled compartment following injection. These proof-of-concept experiments demonstrate that contrast-enhanced 4D MR microscopy of developing frog embryos can effectively visualize key tissue structures and movements during frog neurulation and somitogenesis, complementing cellular level light microscopy of superficial cell layers.

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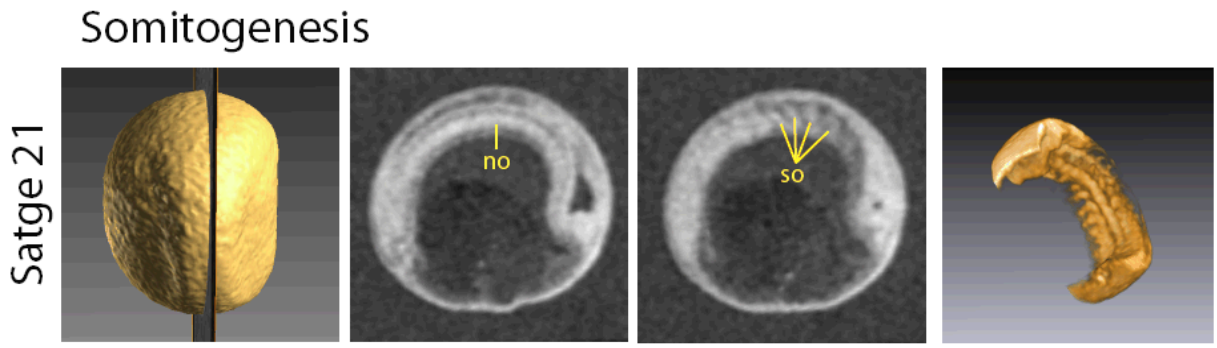


Magnetic resonance microscopy of somitogenesis in a live developing *Xenopus laevis* embryo. The mid-sagittal slice location is shown on an external surface rendering of the embryo (left). The notocord was distinguishable from early neurulation and individual somites are clearly visualized at later stages. Contrast enhancement was limited to animal cells, with intra-embryonic fluid spaces and vegetal cells considerably hypointense. Label key: somites (S0), notocord (N0). Spatial resolution is approximately 19 μm (isotropic) with a temporal resolution of 22 minutes.

354. Digital three-dimensional atlas of quail development using high-resolution MRI

Seth W. Ruffins, Melanie Martin, Scott E. Fraser, Russell E. Jacobs, Rusty Lansford

We have developed a Quail Developmental Atlas (QDA) that provides a perfectly registered volumetric reconstruction of developing quail embryos using a magnetic resonance imaging (MRI) microscope. We are collaborating with curators at the California Science Center and the San Francisco Exploratorium to design and develop interactive exhibits that will bring the QDA to a broader lay audience and around which we intend to develop more formal educational programming. We are in the process of publishing our *ex ovo* (E5-E10) and then *in ovo* (E7-E15) data with TheScientificWorld in an online format for the developmental biology and anatomy research community. We have annotated the gross anatomical structures within the classic spatial compartments comprising the embryo using Amira software. Anatomy will also be used as primary framework for incorporating specific patterns, such as gene expression, the distribution of receptors and their ligands, and cell migration routes.

The QDA project has been greatly advanced with high school students helping to annotate the MRI data sets using Amira software and to produce exhibit quality movies using Final Cut Pro movie editing software. We are updating on a weekly basis and a website has been established to disseminate the QDA (http://atlasserv.caltech.edu/Quail/Start_Quail.html). Our use of high school students also provides several young potential scientists with their first exposure to 'cutting edge' research and direct mentoring from research scientists.

Under current consideration, we propose to develop a publicly accessible educational exhibit. This exhibit would be replicated in at least two institutions and would supplement and enhance existing displays of embryo development with high-resolution interactive imagery. In addition to these public displays, we are collaborating with the Teacher Institute of the San Francisco Exploratorium to bring the QDA to high school class biology, math, and physics classes. The students will be able to carry out 'virtual dissections' of the quail embryos in the biology modules, run MRI-based simulations in the physics modules, and work on numerous "EggMath" and embryo growth math problems in the math modules.

The Teachers Institute at the San Francisco Exploratorium will lead efforts to develop a QDA workshop for high school teachers that will be taught first at the San Francisco Exploratorium in Summer 2007 and then at the California Science Center. We also plan to develop and freely distribute self-contained interactive DVDs that contain QuickTime movies of the QDA that students can easily use, along with the physics and math modules. Our long-term goal is that the website, interactive DVDs and museum exhibits will have a nationwide audience within three years.

The Quail Developmental Atlas represents the interests and intentions of scientists and educators from the San Francisco Exploratorium, the California Science Center, the University of Illinois at Urbana-Champaign, TheScientificWorldJournal, and the California Institute of Technology.

355. Quantitative *in vivo* imaging of the dynamics of gene regulatory networks

Mat Barnet, Scott E. Fraser

The fundamental question of developmental biology is how a single fertilized egg cell gives rise to a complex organism containing thousands, millions, or billions of highly integrated cells. As large-scale genome projects continue to reveal the striking genetic similarities among different species, it is becoming more and more clear that interspecies variation results not from any major differences in the sets of genes different organisms possess, but from differences in the regulation and expression of those genes. To better understand how gene regulatory networks govern embryonic development, we

are collaborating with Eric Davidson's Lab, using sea urchins as a model system. The size, shape, and optical transparency of sea urchin embryos makes them ideal for study by light microscopy. We are developing fluorescence imaging tools and techniques to enable *in vivo* quantification of the dynamics of the gene regulatory network responsible for specification of the sea urchin endoderm and mesoderm. One technique involves quantitatively imaging expression of a reporter gene (e.g., green fluorescent protein (GFP)) in the three-dimensional embryo using confocal laser scanning microscopy (CLSM) or two-photon laser scanning microscopy (TPLSM). By quantifying gene expression patterns in three dimensions over time, we aim to better understand how the dynamics of gene regulatory networks govern developmental processes. Another technique involves the small-molecule fluorophores FlAsH and ReAsH, which recognize and bind tetracysteine tags of the form CCXXCC, which can be engineered into transcription factors and other proteins of interest. Use of FlAsH and ReAsH allows for *in vivo* pulse-chase type experiments, in which we aim to quantify protein production and turnover dynamics. By our quantitative approaches, we hope to generate kinetic data that can be applied to the current, largely qualitative (Boolean), gene network models, to enable the models to make quantitative predictions of regulatory dynamics, thereby helping to further our understanding of how complex organisms develop from single cells.

356. The roles of EphA4 and Ephrin-A5 during cranial neural crest migration in the avian embryo

Carole Lu, Scott E. Fraser

Cranial neural crest cells are multipotent cells that arise in the dorsal neural tube and migrate in discrete streams in the avian embryo. In the posterior hindbrain, the neural crest cells first form a field of cells that then segregate amongst branchial arches 3 and 4. The underlying mechanism of this sorting is unclear. However, this is similar to what occurs in *Xenopus*, where cranial crest cells first migrate as a wave and then segregate to different arches, a process which involves ephrin/Eph signaling. Here we investigate the role of ephrin-A5 and EphA4 in the migration of avian neural crest cells that populate BA3 and BA4.

When we misexpress ephrin-A5, neural crest cells are less likely to populate BA3; other branchial arches are not affected. By labeling the premigratory neural crest cells with a GFP and conducting time-lapse confocal microscopy, we find that ectopic ephrin-A5 affects the direction in which the cells migrate from the neural tube, specifically causing neural crest cells from r6 to delay their migration, and cease migration early.

Misexpression of a constitutively active EphA4 construct causes neural crest cells from r4 and r6 to migrate aberrantly around the otic vesicle. Individual cells display erratic patterns of migration, where they actually turn around after extensive migration and migrate back towards the neural tube. A high level of EphA4 expression also causes premature cessation of migration. Misexpression of a dominant-negative EphA4 construct causes a general reduction in the population of migrating neural crest cells. We also observe mismigration of neural crest cells around the otic vesicle.

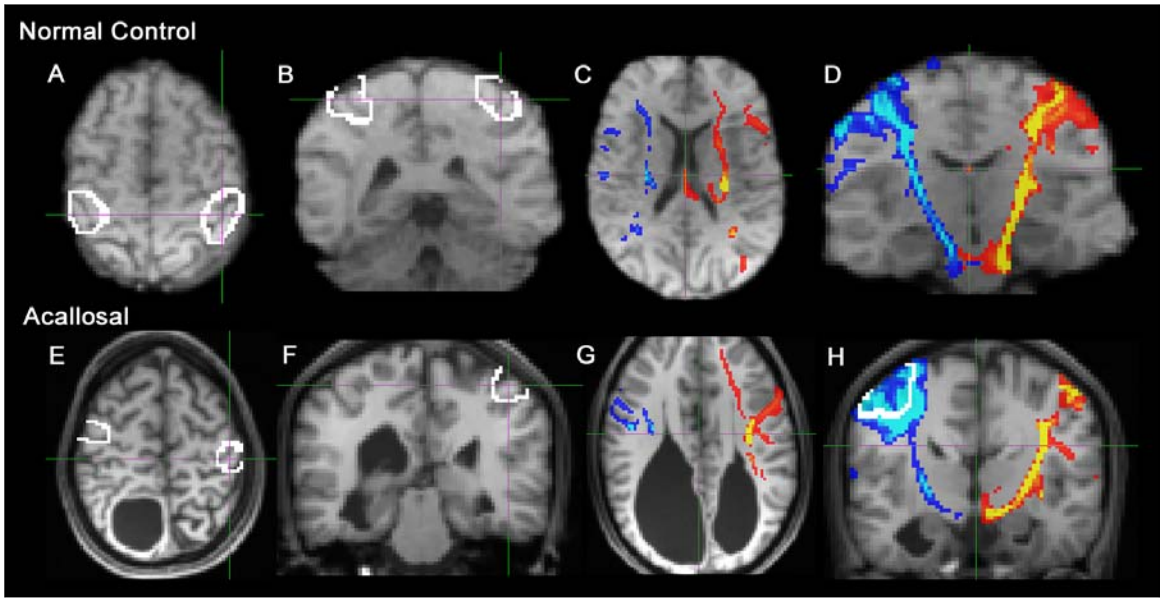
Eph/Ephrin signaling plays a role in ensuring the cranial neural crest cells migrate to their proper branchial arch destination. Surprisingly, the mechanism for this is not limited to sorting of adjacent streams of migrating neural crest cells. Regulation of the cessation and continuation of migration is another potential mechanism.

357. Structural and functional integration in callosal agenesis

J. Michael Tyszka, Lynn Paul[1], Dirk Neumann[2], Matthew Leonard[1], Matthew Bridgman[3], Scott E. Fraser, Ralph Adolphs[1,2]

Agenesis of the corpus callosum (AgCC) is a relatively rare human developmental abnormality in which the corpus callosum is either completely or partially absent. Many adult AgCC subjects have normal IQs and are asymptomatic in routine neurological or psychiatric examinations. As the rate of commissurotomies declines, individuals with AgCC are becoming our primary source of information about callosal transfer in the human brain. AgCC is particularly interesting to those studying network plasticity and compensation since these subjects do not exhibit classical disconnection syndromes. The specific focus of this project is to develop a more complete understanding of long-range structural and functional integration and the compensatory networks associated with AgCC using state-of-the-art non-invasive imaging and advanced statistical modeling. To date we have studied five adult AgCC subjects using a battery of magnetic resonance imaging techniques including BOLD fMRI of coordinated bimanual motor tasks, somatosensory stimulation, high angular resolution diffusion imaging (HARDI) and time-of-flight angiography. Probabilistic fiber tracking (PFT) is now a routine tool for analysis of HARDI data and infers structural connectivity by modeling restricted anisotropic water diffusion within tissues in a non-deterministic framework. We are currently developing methods for integrating PFT with BOLD fMRI in order to characterize the remodeled motor system in AgCC from both the structural and functional standpoints. This project represents a multi-disciplinary, multi-center collaboration between the Divisions of Biology, the Humanities and Social Sciences, Cognitive Neural Systems and the Fuller Graduate School using the advanced resources of the Caltech Brain Imaging Center.

[1]*Division of the Humanities and Social Sciences*
[2]*Computational and Neural Systems*
[3]*Fuller Graduate School, Pasadena*



Integrated probabilistic fiber tracking and BOLD fMRI localization in a normal control and AgCC subject. The left and right primary motor areas were localized using a coordinated finger tapping task (A,B,E,F). Probabistic fiber tracking results are overlaid on coregistered high-resolution structural MRI (C,D,G,H). A total of 5000 fibers were traced for each seed voxel within the motor areas. Exploration of the use of such data as a constraint or prior for connectivity modeling is a key element of this project. Note that in this subject, the occipital horns of the lateral ventricles were significantly enlarged (colpocephaly, visible in axial section in E,G) as were other CSF spaces.

358. Continued development of diagnostic and therapeutic approaches to age-related macular degeneration (AMD)

C.J. Yu, Jeff Fingler, Jon Williams, J. Michael Tyszka

Age-related macular degeneration is the leading cause of vision loss for people over 50 years of age in the Western world. 90% of blindness related with this disease results when sub-retinal neovascularization occurs and damages the macular retina. Current therapies are directed towards treating this form of the disease and halting the progression. As for many diseases, recent studies suggest that treating AMD at early stages is more effective and has better long-term outcomes.

One part of this project is using optical coherence tomography (OCT) to produce depth resolved reflectance imaging of the retina. A commercially available OCT imaging system is currently being adapted to allow imaging of neovascularization much earlier in the disease progression of AMD than possible with current clinical technology.

Before neovascularization occurs, lipid accumulation in Bruch's membrane reduces hydraulic conductivity and macromolecular permeability between the retina and its blood supply. Continued development of MRI-based methods allows accurate measurement of membrane permeability indices using deuterium oxide as a negative contrast agent for 1H MRI. Model-based analysis of fluid equilibration across fresh Bruch's membrane samples shows a consistent increase in membrane permeability following detergent treatment.

359. A modified Golgi impregnation method for *in vitro* MR microscopy

Xiaowei Zhang, Russell E. Jacobs

In the late 19th century, Camillo Golgi revolutionized the budding discipline of neuroscience with his Black Reaction [1, 2], now known as the Golgi stain. The Golgi stain consists of pretreatment with potassium dichromate and other additives [3] followed with silver nitrate resulting in black deposits clearly delineating soma, axons, and dendrites against a yellow background. MR microscopes with different weighting schemes (T1, T2 and proton density weighted, etc.) as well as contrast agents are used in an effort to highlight specific details in a manner analogous to Golgi Black Reaction [4]. Although there is much speculation as to what precisely the Black Reaction does, we hypothesized that, just as pretreatment with $K_2(Cr_2O_7)$ alters tissue properties in a dramatic way for subsequent silver staining, these altered tissue properties (and perhaps locally high concentrations of the Cr(III) paramagnetic) would give rise to different MRI contrast and/or differential absorption of a gadolinium contrast agent and lead to better definition of anatomical features and more facile identification of abnormalities.

Golgi stain MR microscopy augments the MR contrast based on impregnation with potassium dichromate and a Gd-based contrast agent that allows differentiation of the cell layers of the brain (Fig. 1). MR images of brains treated with the four procedures afford qualitatively different contrast (Fig. 2). 3D FLASH images obtained from ProHance plus $K_2(Cr_2O_7)$ treated samples show a high contrast-to-noise ratio and yield superior anatomical

information (Fig. 3). With this new method, anatomical contrast is dramatically improved compared to conventional methods, suggesting potential applications for studies of neurodegenerative diseases through its ability to highlight diverse anatomical regions at high contrast and spatial resolution.

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Figure 1:

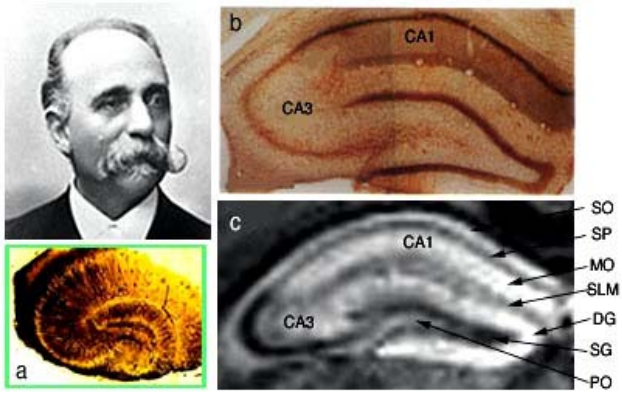

Figure 1. *Photo:* Dr. Camillo Golgi (1843-1926), winner of the Nobel Prize in Physiology or Medicine 1906. a. Hippocampus impregnated by the Golgi stain (an original preparation from Golgi's laboratory kept in the Institute of Pathology of the University of Pavia); b. An example of histologic Golgi stain (*Proc. Natl. Acad. Sci. USA* (1991) **88**(17):7650); c. an expanded MRI view of the hippocampus of the ProHance plus K2(Cr2O7) treated sample. Note the excellent correspondence between the MR microscopy and the histological section of the hippocampus. SO, Stratum Oriens; SP, Stratum Pyramidale; MO, Molecular Layer; SLM, Stratum Lacunosum Moleculare; DG, Dentate Gyrus; SG, Granule Cell Layer; PO, Polymorphic Layer.

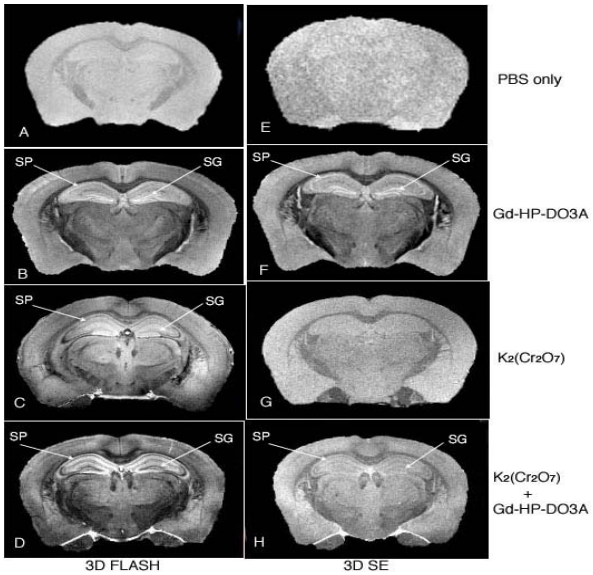

Figure 2: Comparison of MR images of samples with the four soaking procedures. Right column (A-D), 3D FLASH MRI; Left column (E-H), SE T1 weighted MRI. Note hyperintensity in the stratum pyramidale (SP) and Granule Cell Layer (SG) in the Prohance soaked sample (B, F) and black bands in K2(Cr2O7) and ProHance plus K2(Cr2O7) (C, D).

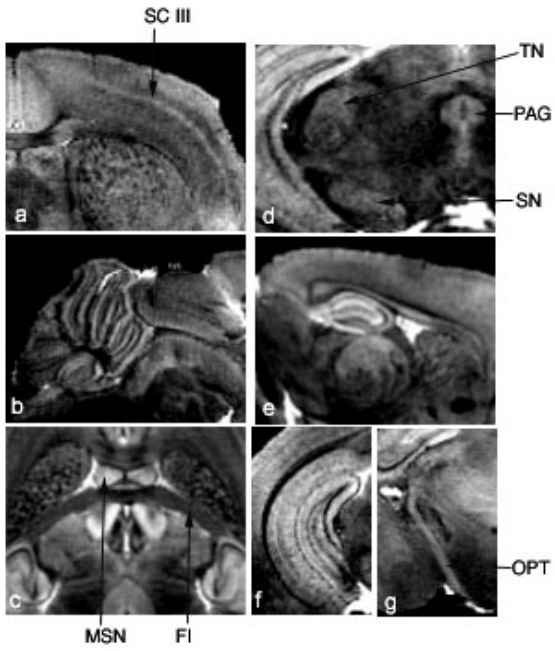

Figure 3. Enlarged views of MR microscopy images (3D FLASH) selected from the brain treated with the modified Golgi method reveal a wealth of anatomic details, such as the basal ganglia, cortex layers, neural tract. SC III, somatosensory cortex layer III; TN, thalamic nucleus; PAG, periaqueductal gray; SN, substantial nigra; OPT, optic track; MSN, medium septum nucleus; FI, fimbria.

360. *In vivo* assessment of interaction between glioblastoma and IL-13 cytolytic T cells

Jelena Zinnanti, Evette Ferkassian, Brenda Aguilar, Michael C. Jensen, Andrew Raubitschek, Russell E. Jacobs

Recent conceptual and technological advances in the field of tumor immunology offer promising therapies for human glioblastoma. To guide the evaluation of clinical trials, non-invasive methods are necessary to follow the effect of treatments. In this work we present parameters of magnetic resonance imaging (MRI) that can be used to monitor the therapeutic action of specific T-cells on glioblastoma *in vivo*. We utilized recently described T cells genetically modified to express a chimeric T-cell receptor with a membrane-bound IL-13 cytokine (zetakine). This zetakine has been shown to bind IL-13 a2 receptors expressed exclusively by high-grade glioblastoma in the central nervous system. Consequently, these zetakine T cells selectively bind and lyse these glioblastoma cells. Our monitoring strategy is based on increased transverse relaxation time (T2) and apparent diffusion coefficient (ADC) following cytolytic action of zetakine T cells on glioblastoma cells. Increased extracellular water as a consequence of glioblastoma cell lysis is reflected as increased T2 values (Figure 1). Cell lysis may also lead to increased extracellular space associated with enhanced water mobility detected as elevated ADC values. Additionally, contrast agent and T1-weighted MRI were used to monitor integrity of the blood-brain barrier (BBB) during zetakine T-cell interaction with glioblastomas. Progression of glioblastomas is associated with increased BBB permeability. Following adoptive transfer of zetakine T cells, partial restoration of BBB integrity is observed (Figure 1, T1-weighted images). These data indicate that zetakine T-cell activity may limit glioblastoma progression including associated BBB disruption. In summary T2 and ADC values can be utilized to monitor zetakine T-cell activity against glioblastomas non-invasively. Furthermore, contrast agents can be used to monitor BBB integrity as an additional indication of glioblastoma progression.

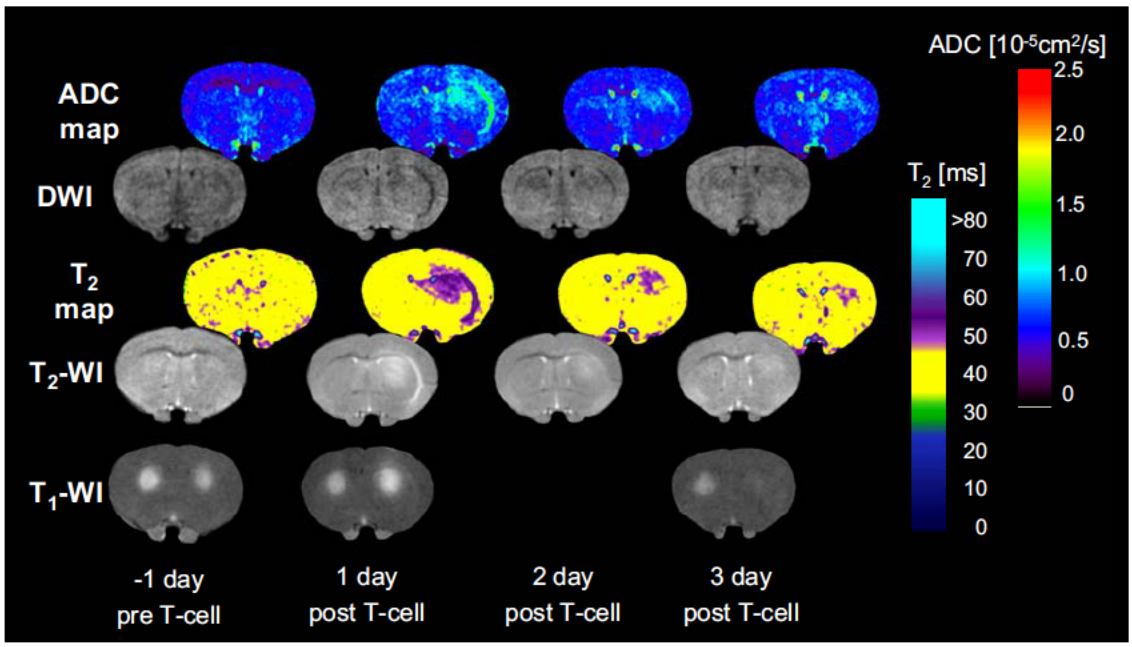

Figure 1. Magnetic resonance images 1-day prior (-1 day) and 1-3 days following right-side T-cell injection. All images are at the level of anterior commissure from the same animal. Glioblastoma cells (line U87) were implanted into the frontal cortex bilaterally 10 days prior to zetakine T-cell transfer. At the bottom, hyperintense signal on contrast enhanced T1-weighted images shows the boundary of glioblastoma and BBB disruption. Note decreased area of hyperintense signal on the right side 3 days after T-cell transfer. Middle and top are pairs of T2-weighted images with corresponding T2 maps, and diffusion-weighted images with corresponding apparent diffusion coefficient (ADC) maps respectively. The highest T2- and ADC-values appeared 1 day post T-cell transfer, and were decreased toward normal at 3 days post transfer. Labeled color bars represent corresponding T2- and ADC- values.

361. Localized IZQ spectroscopy in the live mouse brain using a MR imager

Benoit Boulat, P.T. Narasimhan, Russell E. Jacobs

We have utilized the effects of the distant dipolar field (DDF) [1,2,3] to obtain one-dimensional high-resolution NMR spectra in the full head or a localized region in the brain of live mice. Experiments were conducted on a 11.7T magnetic resonance (MR) imager. The method uses techniques of two-dimensional NMR spectroscopy [4] and can be well described within the formalism known as "Intermolecular Zero Quantum (IZQ) spectroscopy" [5] although a more classical description using the Bloch equations including the DDF would provide for the same physical predictions [6]. The pulse sequences that were used to obtain the spectra were derived from the SEL-HOMOGENIZED class [5,7,8], adding when necessary a localization module. The t_1-modulated signal of the water and of the metabolites in the mouse brain was collected by the RF coil during the time t_2. After 2D Fourier transformation of the time domain data, the projection along the w_1 dimension provided a high-resolution IZQ spectrum exhibiting better immunity against broadening due to field inhomogeneities; indeed in the setting described above, w_1 labels the difference in frequency between the water resonance and the resonances of the metabolites present in the brain of the mice studied.

The figure below shows the *in vivo* 1D IZQ spectrum obtained in the head and a localized region in the brain of a live transgenic mouse. Well-known metabolites are identified in the spectra. Both spectra result from the collection of 128x2048 time domain data points zero filled to 256x4096. Two averages were co-added for the full head spectrum, four for the one localized in a volume of $7x7x7mm^3$ in the brain.

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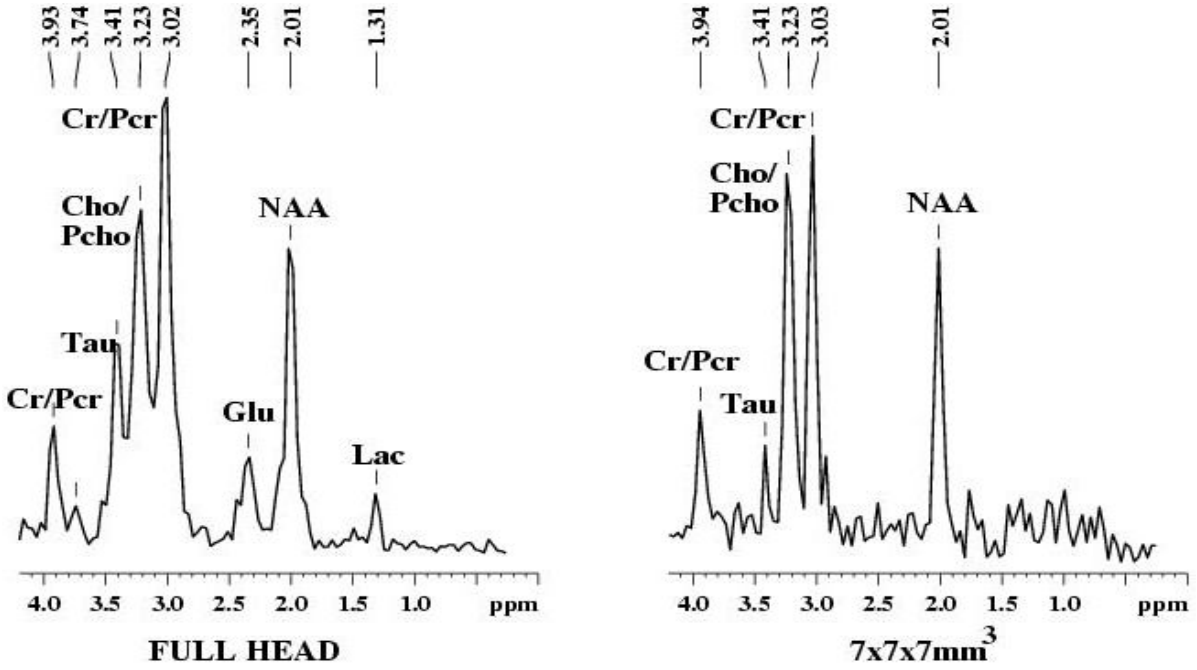

Cr: creatine, Pcr: phosphocreatine, Tau: taurine, Cho: choline, Pcho: phosphocholine, Glu: Glutamate, NAA: N–acetylaspartate, Lac: lactate.

362. Neuronal circuitry by magnetic resonance imaging in animal models of Down's syndrome

Elaine L. Bearer, Xiaowei Zhang, Russell E. Jacobs

The Ts65Dn mouse is a widely used mouse model for human Down's syndrome (DS). DS is caused by a triple dose of chromosome 21 and is the most common genetic cause of mental retardation, affecting ~1/850 live births in the USA. In the fourth decade, DS individuals develop signs and symptoms of Alzheimer's disease (AD). The amyloid precursor protein (APP), a major component of senile plaques of AD, is triplicated on chromosone 21 in DS, thus linking Down's and AD at the molecular level. The Mobley lab at Stanford has shown that transport of NGF, a growth factor for cholinergice neurons, may be defective in DS and AD.

Professor Bearer's laboratory at Brown University found that herpesvirus (HSV), the cause of the common cold sore, physically interacts with APP (Satpute-Krishnan, DeGiorgis *et al*., 2003). Using novel nano-bead technology, it was also found that APP is sufficient for anterograde transport. These discoveries raise the alarming possibility that HSV might be a risk factor for Alzheimer's disease.

To test this possibility, we are taking several approaches, one of which is to study the pathways into and within the brain that the HSV virus might follow. With contrast agents such as manganese, MRI can reveal circuitry within the brain of living mice (Pautler, *et al*., 2003), tracking brain circuitry and also allowing us to follow the course of a disease over time.

We have used MRI to image relevant pathways in the living brain of an animal model of human Down's syndrome, the Ts65Dn mouse. Development of this technology is expected to transcend applications to the problem of DS and AD, and provide insights about human thought, behavior, and emotion--and even musical appreciation.

Methods: Mice were injected with 3-5 nL of 200 mM $MnCl_2$ into the hippocampus and imaged by MR before and at 30 min, 6 hr and 24 hr afterwards. An 11.7T 89 mm vertical bore Bruker BioSpin Avance DRX500 scanner (Bruker BioSpin Inc, Billerica, MA) was used to acquire *in vivo* mouse brain images with a 35 mm linear birdcage RF coil. We employed a 3D RARE imaging sequence with RARE factor of 4, 4 averages, TR/TE_{eff} = 300ms/21ms; matrix size of 256x160x128; FOV 23 mm x 14.4 mm x 11.5 mm; yielding 90 mm isotropic voxels with 102 minute scan time. A total of 13 mice (7 Ts65Dn and 6 wild-type littermates) were imaged**.** Pre-injected brain images were averaged using full nonlinear normalization. The nonlinear transformation was used to drive each individual mouse's pre-injected scan into a template of all scans and was then used to drive each mouse's 0.5 hr, 6 hr, and 24 hr scan into the same template. Anatomical differences between the Ts65Dn and wild-type brain precluded successful nonlinear normalization into the same template. Students' t-test on a voxel-by-voxel basis determined differences with $P<0.005$ between sets of brain images.

Results and Interpretation: Statistical parametric mapping comparisons of the seven averaged DS brains with averaged littermate controls demonstrated a statistically significant difference in the extent of Mn^{2+} transport. In Ts65Dn mice, the Mn^{2+} travels further faster, being detectable in the medial septal nucleus, nucleus accumbens and other anterior brain structures at 6 hr after injection into the hippocampus in Ts65Dn brains but not in littermate controls. These results suggest that transport is increased in DS. This is further data supporting a relationship between intraneuronal transport and cognition, and provides us with tools to explore HSV transport and its role in AD.

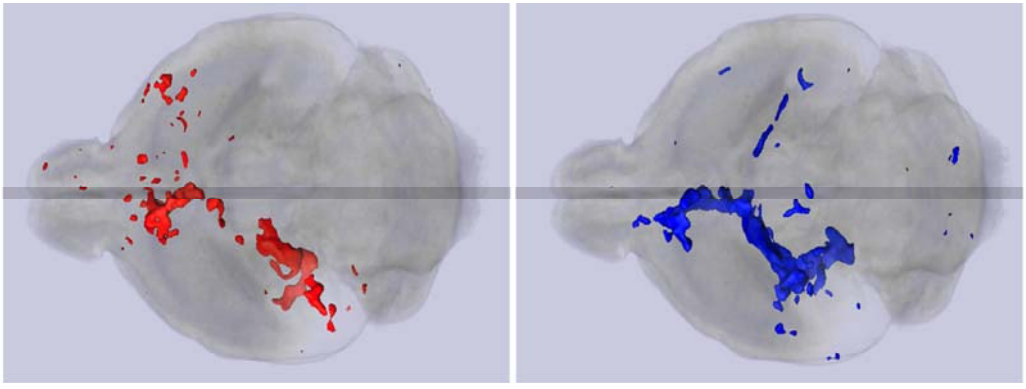

Figure: Mn^{2+} transport is accelerated in a mouse model of Down's syndrome. Littermate controls (left side) and Ts65Dn (right side) mice. Gray background shows brain anatomy and colored voxels denote where Mn^{2+} is transported by 6 hrs post injection.

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363. Applications of Terahertz imaging to medical diagnostics

Peter H. Siegel, Warren Grundfest (UCLA), Scott E. Fraser

This research program aims to apply terahertz (THz) source and sensor technology towards (1) establishing and cataloging properties and contrast mechanisms in biomaterials and tissues and (2) investigating potential disease diagnostic applications. THz waves occupy the wavelength region between 100 and 1000 microns, well beyond the traditional infrared. As a consequence they can penetrate more readily into many optically opaque materials including plastics, wood, clothing, some crystalline structures and, as is the emphasis for this program, a small number of biomaterials – lipids, bone, skin, hair, etc. Unlike infrared and optical signals, THz penetration in tissue is dominated by absorption not scattering, making the contrast mechanisms very different than those that are already being exploited in the optical regime. Water absorption is also extremely high (=100-300 cm^{-1}) which has the disadvantage that very strong THz signal sources are required for significant tissue penetration, but the advantage that very subtle changes in fluid content are detectable (the basis for disease diagnostics). The first successful THz imaging systems have been based on picosecond pulsed-laser time domain spectroscopy techniques. These instruments offer unique spectral and time resolved information content but have limited penetration depth (tens of microns in tissue) and only modest signal-to-noise ratio (100-1000). This program has now demonstrated three types of THz imaging systems based on continuous wave and swept frequency heterodyne techniques (more generally used in space science and communications). One system operates at a fixed frequency of 2520 GHz (120 microns wavelength) and boasts a signal-to-noise of more than 10^{10}. A new frequency agile system, established last year, uses a millimeter wave vector network analyzer (MVNA) and can image between 100 and 1000 GHz (3 mm to 300 microns wavelength). Finally, this year a swept frequency instrument, employing active illumination of the sample and real-time transmission, reflection and/or scattering measurements over 15% bandwidth can perform both index of refraction or narrow band spectroscopy measurements on inserted samples between 550 and 650 GHz. This system uses all solid-state components, has hand held transmit and receive heads and portable electronics and power supplies. It is controlled by a lap top computer. Current work will extend this swept system so as to enable imaging and time of flight (range finding) capabilities.

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P.H. Siegel and R.J. Dengler, *Terahertz Heterodyne Imaging: Instruments*, to appear in International Journal of Infrared and Millimeter Waves, summer 2006.

364. Flexible ribbon guide for *in vivo* and hand-held THz imaging

Peter H. Siegel, Cavour Yeh, Scott E. Fraser*

Recent interest in terahertz frequency imaging for medical applications (wavelength range from 1 mm to 100 microns) has resulted in a flurry of new instrument proposals using both time domain and high-resolution frequency domain spectral techniques. However, to date, there exists no methodology for transporting terahertz signals from place-to-place with low loss, other than rigid-path free-space quasi-optical beam propagation. In order to take advantage of modalities common at optical wavelengths, including *in vivo* and portable hand-held sensor/receiver systems, the equivalent of signal-confining optical fiber links must be developed for the far infrared and terahertz bands. Commonly employed transparent materials in the visible (i.e., amorphous glasses or clear plastics) are all extremely lossy at longer wavelengths due to strong vibrational mode absorption. Dielectric substances with low absorption coefficient and high index do exist at terahertz frequencies, but they tend to be crystalline (quartz, silicon, germanium, GaAs) and therefore have poor mechanical properties when it comes to forming flexible guiding structures. Metallic waveguide (hollow or coaxial), although somewhat flexible, has untenable high resistive wall loss. A few plastics such as Teflon, polyethylene and polypropylene, have very low dispersion and absorption but have a low refractive index that makes it difficult to confine single-mode terahertz energy as it propagates around bends or through joints. Earlier work by our collaborators has shown that high index materials formed into ultra-thin ribbons can form very low loss guiding media at millimeter-wave frequencies (30-100 GHz). Extrapolating this concept into the terahertz bands, and taking advantage of modern fabrication techniques, we believe it is possible to use a combination of high-dielectric-constant crystalline materials in conjunction with low-loss, low-index plastics to produce the equivalent of flexible terahertz optical fiber, i.e., "terahertz ribbon guide." Such a development would allow terahertz instruments to be freed from their fixed-beam-path table top environment, enabling, for the first

time at these wavelengths, both hand-held scanner and *in vivo* endoscopic applications. This task has been investigating both the design and fabrication of low-loss single mode terahertz guiding structures. This year we have developed a hybrid concept which combines the concept of thin crystalline ribbon guide with a surrounding sheath of thin tubular quartz, that both holds the ribbon in place and serves as a dielectric shield to contain the propagating fields. The new structure has a cross section that resembles a "DO NOT ENTER" sign (below) and therefore has been affectionately designated Do Not Enter guide. A prototype is now in production using drawn quartz fiber and will be evaluated this year.

[*]*California Advanced Studies*

This work is being funded by an NIH K25 training grant held by Dr. Siegel, who holds a joint appointment as a Technical Group Supervisor for Submillimeter Wave Advanced Technology (SWAT) at JPL.

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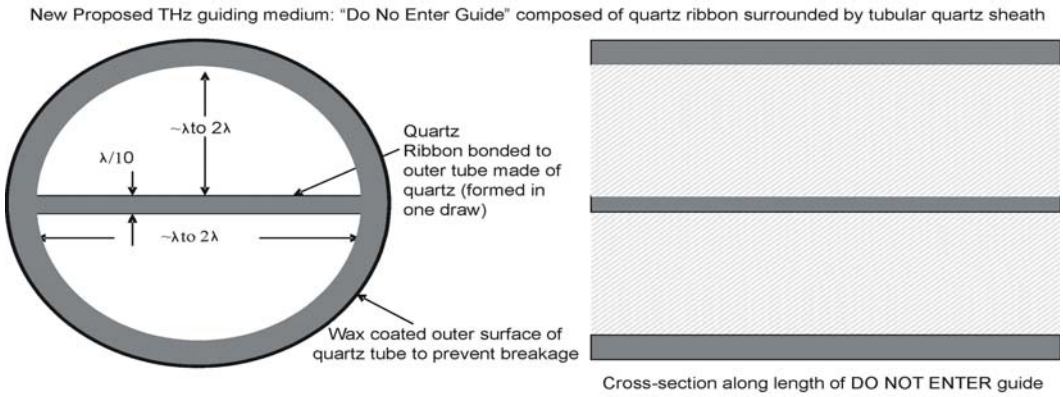

365. Nanobased signaling biosensors

Periklis Pantazis, Ye Pu, John Hong[*], Demetri Psaltis, Scott E. Fraser

Signaling regulates embryonic development by providing positional information to cells so that they differentiate properly as they proliferate to build up the final shape. However, defects leading to abnormal activation of signal pathways often underlie most tumorigenic events. In recent years, much has been discovered about the molecular and biochemical characteristics of a variety of signal transduction pathways. In contrast, the cell biology of such signaling events is starting now to be studied. In particular, we are interested in the following questions: 1) when does signaling occur; 2) where does it take place; and, 3) for how long? We aim to address these questions by developing biosensors for following *in vivo* signaling events with large sensitivity increase and high spatial and temporal resolution. We plan to use nanobased biosensors that monitor signal transduction through visualization of conformational changes and protein-protein interaction of components of signaling pathways. We aim to generate reagents that will enable us to follow various signaling events in real time. Ultimately, the established biosensors will be used to investigate signaling during zebrafish development and in the manifestation of cancer.

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366. Formation of self-assembled monolayers on gold

Christie A. Canaria, Scott E. Fraser, Rusty Lansford

Self-assembled monolayers (SAMs) are used to tune the chemical properties of solid surfaces. Specifically, we use functionalized alkylthiols to modify Au substrates. These monolayers may be used to create surfaces that specifically bind proteins, oligos, and cells.

We have demonstrated that alkylthiols modified with biotin specifically bind avidin and avidin-like proteins. Mixed monolayers of PEG- and biotin-modified alkylthiols were generated on gold and characterized by fluorescence microscopy (Figure 1). In addition, oligo-modified alkylthiols have been designed and synthesized. Oligos were synthesized as 26mers with a BamHI cleavage site in the middle of the sequence. Hybridization of fluorescent compliment strands was then monitored using fluorescence microscopy. Incubation of the substrate with the enzyme BamHI results in cleavage of the double-stranded oligo and the attached fluorophore (Figure 2). Lastly, we are modifying gold and silicon substrates for the growth and culture of mammalian cells. Yolk sac endothelial cells[2] can be grown on a number of different substrates including bare- and monolayer-coated gold (Figure 3). We are currently investigating the quality of various SAMs for the purpose of growing cells with the

intent of culturing cells on spatially encoded and defined substrates.

References

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(2) Generous gift form Professor Charlie Little, University of Kansas.

List of Figures

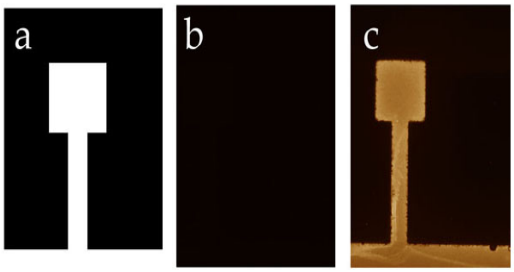

Figure 1. Streptavidin binds to biotinylated surfaces. a) Cartoon image of a gold pad on a silica substrate. b) Triethylene glycol monolayer-modified surface does not bind Cy3-labeled streptavidin. c) Biotinylated monolayer-modified surface binds Cy3-labeled streptavidin. The background silica surface does not.

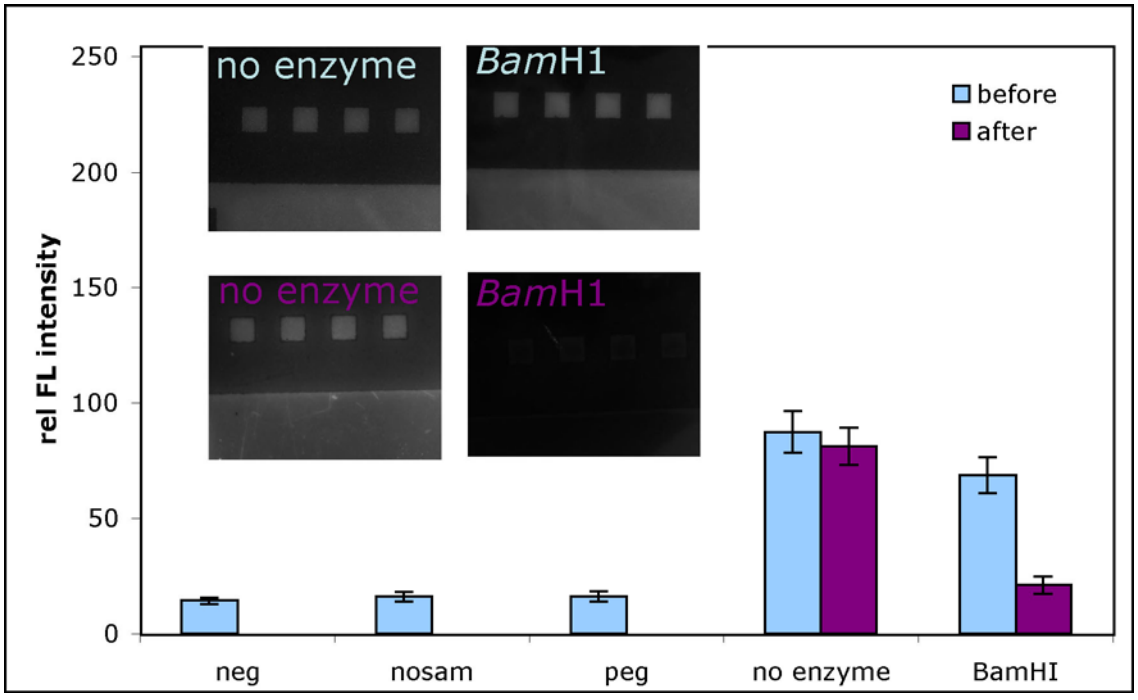

Figure 2. Graphed plot of fluorescence intensities for labeled double-stranded oligos on Au pads before and after enzymatic cleavage by *Bam*HI. Inset, microscopy images for control sample (left) and *Bam*HI treated sample. n= 16 samples.

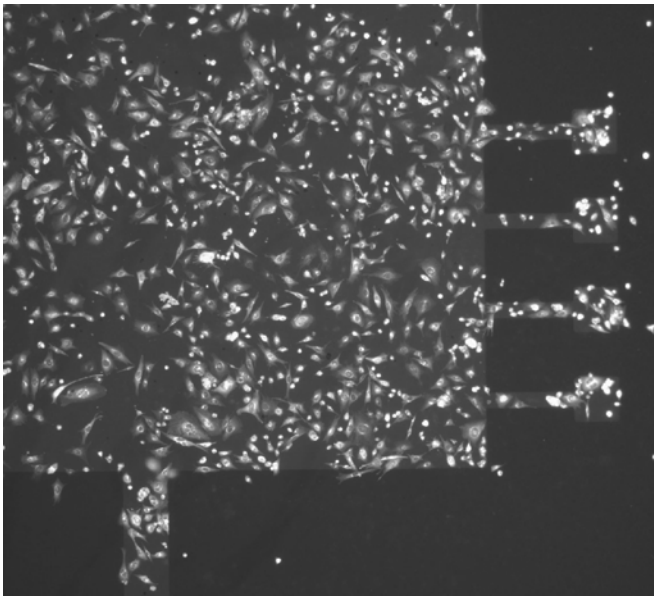

Figure 3. YSE cells spread and adhere onto photo-patterned bare gold surfaces, but do not adhere to PEG-treated silicon. Cells stained with BODIPY-ceramide (Molecular Probes).

367. Engineering a fluorescent reporter of integrin linked kinase activity

Wei Shen, Scott E. Fraser

Integrin linked kinase (ILK) is a critical regulator of cell adhesion and migration, and is involved in many aspects of animal development such as angiogenesis and axon growth. To study the temporal and spatial dynamics of ILK activity in living cells, we engineered a genetically-encoded fluorescent biosensor. The biosensor consists of fusions of cyan fluorescent protein (CFP), a substrate for ILK, a binding domain for phosphorylated substrate, and yellow fluorescent protein (YFP). When ILK is activated, the substrate is phosphorylated and interacts with the binding domain, bringing CFP close to YFP. The fluorescence resonance energy transfer signal from CFP to YFP provides the information of ILK activity.

368. Characterization and visualization of the FcRn-dependent transcytotic pathway using high-resolution fluorescence confocal microscopy

*Galina V. Jerdeva, Devin B. Tesar, Scott E. Fraser, Pamela J. Bjorkman**

Specific delivery of proteins across polarized epithelia is controlled by receptor-mediated transcytosis. Based on studies of the trafficking of model receptors such as the polymeric immunoglobulin receptor (pIgR), the pathways for receptor-mediated transport of protein ligands in the basolateral to apical direction are relatively well understood. Less is known about the trafficking pathways for receptors that transport from the apical to basolateral surface. One such receptor is the neonatal Fc receptor (FcRn), which transports maternal immunoglobulin G (IgG) across intestinal or placental epithelial barriers to provide immunity to fetal or newborn mammals and serves as a protection receptor for IgG, thereby increasing its serum half-life. To investigate FcRn-mediated transport of IgG, we are using Madin-Darby Canine Kidney (MDCK) cells stably expressing FcRn (MDCK-FcRn). The transfected cells specifically transcytose IgG and Fc across polarized cell monolayers, therefore presenting an *ex vivo* system that mimics the *in vivo* FcRn-dependent transport system. Using high-resolution confocal and multiphoton microscopy, we will identify intracellular compartments involved in transcytosis of labeled Fc and IgG in MDCK-FcRn cells by colocalization studies with organelle-specific markers. Comparing the intracellular trafficking of FcRn and its ligands with trafficking of a basal to apical model receptor, pIgR, which transports dimeric dIgA (dIgA), will provide information about common and specific trafficking pathways of IgG versus dIgA. To compare FcRn- and pIgR-dependent intracellular pathways**,** we will generate MDCK cells stably expressing both receptors (MDCK-FcRn-pIgR). Fluorescently-labeled Fc and dIgA will be internalized apically and basolaterally, respectively, by polarized MDCK-FcRn-pIgR monolayers, and the intracellular locations of internalized ligands will be investigated by confocal or multi-photon microscopy. To get more detailed information about the dynamics of Fc trafficking and the identities of trafficking compartments, real-time high-resolution microscopy will be performed on living polarized cells using highly efficient, photo-bleaching resistant fluorescent probes such as Alexa-dyes and Quantum Dots.

**Professor, Division of Biology, Caltech*

369. Single-biomolecule resolution imaging with an optical microscope

Lawrence A. Wade, C. Patrick Collier[*] Scott E. Fraser

A Fluorescence Apertureless Near-field Scanning Optical Microscope (FANSOM) has been developed with FWHM optical resolution below 10 nm when imaging at ~600 nm wavelengths [1]. The apparatus combines an epi fluorescence optical microscope and an atomic force microscope (AFM) to obtain single-molecule sensitivity and optical resolution limited by the sharpness of the AFM probe. The AFM probe is used to stimulate or reduce the detected fluorescence emission rate depending on the AFM tip material and the polarization of the excitation light. The probe-sample interaction is described by near-field dipole-dipole physics, resulting in a stimulated emission rate that varies by r^6. When tapping the probe over the substrate being imaged, the near-field component is sharply modulated at that tapping frequency, thereby enabling separation from the far-field background during post-processing. Images of fluorescent single molecules taken in a physiological environment will be presented.

We are also developing probe and substrate technologies to enable FANSOM to image and time-resolve the dynamics of biomolecular interactions. The tools developed include generalized techniques for the growth and attachment of nanotubes for use in AFM imaging. With our nanotube tips we have generated 0.5 nm resolution AFM images, potentially enabling optical imaging of single molecules with resolutions approaching 1 nm [2]. These nanotube probes can be uniquely functionalized at their tips, serving as the foundation of an effort to develop single-molecule sensors. Coating these probes with nm-thick conformal fluorocarbon films has enabled the fabrication of molecular-scale electrical probes [3]. We have also developed silane-chemistry dip-pen nanolithography techniques for patterning glass coverslips with functional proteins, peptides, aptamers, etc. [4,5]. By combining FANSOM with these techniques we anticipate patterning functional biomolecules onto glass coverslips and then individually probing highly specific molecular interactions at *in vivo*-like molecular concentrations and characterizing cellular contents and expression with single-molecule discrimination. Finally, techniques are being developed for patterning phospholipid bilayers for use as model membrane systems. Together, these tools should prove particularly well suited for probing bio-interface problems such as viral insertion, transmembrane protein triggering and lipid raft formation and function.

[*]*Department of Chemistry*

Publications

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Summary: We are interested in multiple questions in basic and applied insect biology. For further information on Hay lab research consult our web page (http://www.its.caltech.edu/~haylab/). One goal of our work is directed towards understanding the genetic and molecular mechanisms that regulate cell death, proliferation, innate immunity and spermatogenesis. We use *Drosophila melanogaster* as a model system to identify genes that function to regulate these processes. Important cellular regulatory pathways are evolutionarily conserved; thus, molecules identified as regulators of these processes in *Drosophila* are likely to have homologs in vertebrates and the pathways that link these molecules are likely to be regulated similarly. A second goal of our work is to develop transgenic insects that will prevent transmission of mosquito-borne diseases such as malaria and Dengue fever. More than 500 million people are infected with the malaria parasite each year, resulting in more than 1 million deaths. Dengue, a mosquito-borne virus infects more than 100 million people each year, resulting in more than 25,000 deaths. Effective vaccines for these diseases do not exist, and in the case of malaria, the causative agent, the parasite *Plasmodium falciparum* has acquired resistance to many drugs. Vector suppression through the release of sterile males, the use of insecticides, or modification of the environment provides an important tool for limiting mosquito-borne disease. However, each approach has limitations. Release of sterile males provides only transient population suppression, insecticides affect many non-target species and mosquitoes often evolve resistance to these compounds, and wholesale modification of the environment may not be feasible, or desirable in some situations based on ecological concerns. Our goals are two-fold, to develop transgenic insects that lack the ability to transmit these pathogens, and to develop genetic tools for driving these genes into wild populations of insects, thereby blocking disease transmission.

370. *Drosophila* models of human neuro-degenerative diseases

Ming Guo (and the Guo lab), Bruce A. Hay

In collaboration with the Guo lab at UCLA we are studying *Drosophila* models of the two most common neurodegenerative diseases, Alzheimer's disease and Parkinson's disease.

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371. Gene activation screens for cell death regulators: MicroRNAs, small non-coding RNAs, define a new family of cell death regulator

Chun Hong Chen, Haixia Huang

We have carried out several screens for cell death regulators in the fly and have identified a number of new molecules. Among these are multiple microRNAs, small noncoding RNAs that function by inhibiting translation of target transcripts*. We are interested in determining when and where these molecules regulate death, as well as the nature of their targets. We are also designing microRNAs that target known cell death regulators as a way of probing the function of these proteins in specific contexts.

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372. Cell death, caspases and IAPs

Israel Muro, Jun Huh, Soon Ji Yoo, H. Arno J. Müller

In flies and vertebrates most, if not all, cells can undergo apoptosis in the absence of new gene expression, indicating that the components required to carry out apoptosis are present and ready for activation. The core of the cell death machine consists of members of a family of proteases known as caspases, which become activated in response to many different death signals. Active caspases then cleave a number of different cellular substrates that ultimately lead to cell death and corpse phagocytosis. Most if not all cells constitutively express caspase zymogens (inactive precursors) sufficient to bring about apoptosis. Thus, the key to cell death and survival signaling revolves around controlling the levels of active caspases in the cell. Several basic strategies are used to regulate caspase activity, and the core proteins that drive caspase-dependent death are evolutionarily conserved. In *Drosophila* many cells experience chronic activation of the apical cell death caspase Dronc. If unrestrained, active Dronc cleaves and activates downstream effector caspases that bring about cell death. Cells survive because they express the IAP DIAP1, which suppresses Dronc activity, as well as that of caspases activated by Dronc. One major pathway through which caspase-dependent cell death in

flies is induced is through the regulated expression of pro-apoptotic proteins that disrupt DIAP1-caspase interactions through several different mechanisms, each of which has the effect of unleashing a cascade of apoptosis-inducing caspase activity. We are interested in several questions. 1) What are the signals that lead to caspase activation in cells that would normally live? 2) How do IAPs regulate caspase activity and when and where does this regulation define points of control? 3) How is IAP activity regulated? 4) And finally, as discussed further below, how do caspases, IAPs and their regulators work to regulate non-apoptotic processes? We are using both genetic screens and biochemical approaches to identify the critical molecules.

373. Maintaining tissue size: the role of death-induced compensatory proliferation

Jun Huh

Achieving proper organ size requires a balance between proliferation and cell death. Tissues often experience stresses that lead to ectopic death. In order for normal development to occur, this death must be followed by compensatory proliferation that fills in the gaps. We were interested in exploring the idea that it is the dying cell itself that sends a signal to neighbors, driving their proliferation. We tested this hypothesis in several ways. In brief, we found that activation of the apical caspase Dronc, activity of which is required for many cell deaths in the fly, is both necessary and sufficient to drive compensatory proliferation in neighboring cell*. Interesting questions for the future include: 1) What are Dronc's proliferation targets? 2) Are there other contexts in which caspases function in a sense non-autonomously to alter cell fate or behavior?

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374. Cell death in the fly eye

Jeffrey Copeland

Echinus is a *Drosophila* mutant that lacks normally occurring cell death in the eye. We cloned the *echinus* gene and found it encodes a member of an evolutionarily conserved family of proteins, the USPs, which remove ubiquitin from proteins. Echinus lacks interactions with known cell death regulators, suggesting that it acts at a novel point to promote death.

375. Caspases and their regulators in a non-apoptotic process, spermatid differentiation

Jun Huh, Jean Edens

We have found that multiple caspases, acting through distinct pathways, acting at distinct points in time and space, are required for spermatid individualization, a process in which spermatids (which develop in a common cytoplasm) become enclosed in individual plasma membranes and shed most of their cytoplasm*. Spermatid individualization is an evolutionarily conserved process, but little is known about how it is brought about. Several questions are of interest to us: 1) What are the sources of the caspase activity (what are the upstream signals)? 2) What are the nonapoptotic targets that facilitate differentiation? 3) How is cell death prevented in the face of high levels of caspase activity that would normally be associated with cell death? 4) Do caspases play similar roles in promoting spermatid differentiation in mammals?

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376. Cell death and the innate immune system

Jun Huh, Israel Muro, Chun Hong Chen, Ian Foe, Soon Ji Yoo, Jin Mo Park, Ming Guo

As discussed above, many IAP family proteins inhibit apoptosis. IAPs contain N-terminal BIR domains and a C-terminal RING ubiquitin ligase domain. *Drosophila* DIAP1 protects cells from apoptosis by inhibiting caspases. Apoptosis initiates when proteins such as Reaper and Hid bind a surface groove in DIAP1 BIR domains via an N-terminal IAP-binding motif (IBM). This evolutionarily conserved interaction disrupts IAP-caspase interactions, unleashing apoptosis-inducing caspase activity. DIAP2 overexpression also inhibits Rpr- and Hid-dependent apoptosis, but little is known about DIAP2's normal functions. We generated *diap2* null mutants, which are viable and show no defects in developmental or stress-induced apoptosis. Instead, DIAP2 is required for the innate immune response to Gram-negative bacterial infection. DIAP2 promotes cytoplasmic cleavage and nuclear translocation of the NF-κB homolog Relish, and this requires the DIAP2 RING domain. Increasing the genetic dose of *diap2* results in an increased immune response, while expression of Rpr or Hid results in down-regulation of DIAP2 protein levels*. Together these observations suggest that DIAP2 can regulate immune signaling in a dose-dependent manner, and that DIAP2 is regulated by IBM-containing proteins. Therefore, *diap2* may identify a point of convergence between apoptosis and immune signaling pathways.

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377. Driving genes for disease refractoriness into wild pest insect populations

Chun Hong Chen, Haixia Huang, Catherine Ward, Jessica Su, Bruce Hay

An attractive approach to suppressing mosquito-borne diseases involves replacing the wild-insect population with modified counterparts unable to transmit disease. Mosquitoes with a diminished capacity to transmit *Plasmodium* have been identified in the wild and created in the laboratory, demonstrating that endogenous or engineered mosquito immunity can be harnessed to attack *Plasmodium*. However, a critical unanswered question is how to spread these effector genes throughout the areas

inhabited by disease-transmitting insects. Epidemiological and modeling studies suggest that it will be necessary to rapidly replace a large percentage of the wild mosquito population with refractory insects in order to achieve significant levels of disease control. Because insect disease vectors are spread over wide areas and can migrate significant distances, mass release of refractory insects associated with simple Mendelian transmission of effector-bearing chromosomes is unlikely to result in a high enough frequency of transgene-bearing individuals. Compounding this problem, enhancement of immune function in insects is often costly, requiring tradeoffs with other life history traits such as longevity and fecundity. Transgenesis itself may also carry some fitness cost. Therefore, it is likely that insects carrying effector transgenes will be less fit than their wild counterparts, resulting in a decrease in the fraction of individuals carrying genes for refractoriness over time. These observations argue that population replacement will require coupling of genes conferring disease refractoriness with a genetic mechanism for driving these genes through the wild population at greater than Mendelian frequencies.

Maternal-effect selfish genetic elements are attractive candidates to mediate drive because when present in a female, they must be inherited in the next generation in order for the offspring to survive. This behavior is predicted to lead to rapid spread of the element within the population even if it carries an associated fitness cost because the chromosome that carries it gains a transmission advantage relative to counterparts that do not. We have engineered novel maternal-effect selfish genetic elements in *Drosophila* that can drive population replacement and that are resistant to recombination-mediated dissociation of drive and effector functions. The genetic and cell-biological principles utilized should be generally applicable to a number of other species and have the potential to allow for iterative cycles of population replacement. We are now interested in expanding this work in *Drosophila* into the mosquito system.

378. Sensing and killing dengue and yellow fever virus-infected cells in their insect host

Kelly J. Dusinberre

Dengue and yellow Fever virus infect mosquitoes during a blood meal. The virus must first enter and replicate inside mosquito midgut cells in order to be transmitted to a new individual during a subsequent blood meal. Our goal is to develop transgenes that are phenotypically neutral when expressed in uninfected individuals, but that kill virus-infected cells. The virus encodes several activities that are not present in uninfected host cells. These include a viral polyprotein protease, and RNA-dependent RNA polymerase. We are developing molecules that sense these activities and kill cells in which they occur.

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Summary: This year, as in the past, we continue to work toward the answers to some of the fundamental questions raised by plant growth and development. We also continue to use as our experimental subject the small mustard *Arabidopsis thaliana*, which is to plant science what the fruit fly is to genetics, or the mouse to mammalian research – an organism with numerous advantages for use in the laboratory, that in its biology is representative of a much larger group of organisms, for which it can serve as an experimental proxy. The questions of development that we are approaching are largely those of pattern formation – how does a single cell like an egg, or a small group of cells like a flower primordium or shoot apical meristem, produce a much larger and much more organized structure - a plant, flower, or a shoot?

Much of our work centers around the shoot apical meristem, the small group of stem cells at the tip of each shoot, that provides the cells for stem, leaf, and flower. We have developed a set of live-imaging methods so that we can follow the cell divisions, patterns of gene expression, and subcellular locations of regulatory proteins in the meristem in real time. We have also developed a series of methods to perturb meristem function – by turning on or off specific regulatory genes and proteins, or by deleting cells or groups of cells. By combining visualization and alteration, we are inferring the mechanisms by which cells in the meristem learn their positions, and thereby act so as to produce patterned structures. We are also using these methods to study meristematic self-assembly. Culture plant cells form disorganized masses called callus, and regions of callus self-organize to form new meristems, which give rise to new shoots. By observing the details of this process, we are hoping to learn its rules, and to understand how completely unpatterned tissue can make highly patterned collections of stem cells.

Similar methods are being applied to flower development, to find the processes that regulate the genes that specify floral organ identity, that allow cells to remember their state of gene activity through divisions, and that specify individual cell types such as the giant cells of the sepals. In addition, we are taking novel molecular approaches to flower development – study of a family of regulatory RNA molecules that act in allowing each flower on the plant to have the correct number of floral organs (as *Arabidopsis* flowers normally have four sepals, four petals, six stamens, and two carpels), and experiments to determine the complete set of gene activities that are characteristic of the development of early flowers, and of each type of floral organ.

Finally, there is a set of experiments that follows upon the recognition that the *Arabidopsis* genome contains a family of genes related to bacterial stretch-activated ion channels. These experiments, using the methods of genetics and physiology, are designed to reveal how it is that plant cells respond to osmotic changes.

379. Auxin transport patterns and primordium development

M.G. Heisler, C. Ohno, P. Das, P. Sieber, G.V. Reddy, J.A. Long[*]

Plants produce leaf and flower primordia from a specialized tissue called the shoot apical meristem (SAM). Genetic studies have identified a large number of genes that affect various aspects of primordium development including positioning, growth, and differentiation. So far, however, a detailed understanding of the spatio-temporal sequence of events leading to primordium development has not been established. By using confocal imaging of green fluorescent protein (GFP) reporter genes in living plants we have analyzed the expression and polarity of PINFORMED1 (PIN1), the auxin efflux facilitator, and the expression of the auxin-responsive reporter DR5. We find that PIN1 undergoes stereotypical polarity changes which, together with auxin induction experiments, suggest that cycles of auxin build-up and depletion accompany, and may direct, different stages of primordium development. Imaging of multiple GFP-protein fusions shows that these dynamics also correlate with the specification of primordial boundary domains, organ polarity axes, and the sites of floral meristem initiation. These results provide new insight into auxin transport dynamics during primordial positioning and suggest a role for auxin transport in influencing primordial cell type.

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380. An auxin gradient model for plant phyllotaxis

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Recent studies show that plant organ positioning may be mediated by localized concentrations of the plant hormone auxin. Auxin patterning in the shoot apical meristem is in turn brought about by the subcellular polar distribution of the putative auxin efflux mediator, PIN1. However, the question of what signals determine PIN1 polarization and how this gives rise to regular patterns of auxin concentration remains unknown. We have addressed these questions by using mathematical modeling combined with confocal imaging. We propose a model that is based on the assumption that auxin influences the polarization of its own efflux within the meristem epidermis. Such a model is sufficient to create regular spatial patterns of auxin concentration on systems with static and dynamic cellular connectivities, the latter governed by a mechanical model. We also have optimized parameter values for PIN1 dynamics by using a detailed auxin transport model, for which parameter values are taken from experimental estimates, together with a template consisting of cell and wall compartments as well as PIN1 concentrations quantitatively extracted from confocal data. The model shows how polarized transport can drive the formation of regular patterns.

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381. Local signals controlling PIN1 polarity in the shoot apical meristem

Marcus G. Heisler, Carolyn Ohno

Previously we have proposed that auxin transport patterns in the shoot meristem are coordinated via auxin gradients between neighboring cells. This model assumes that relative auxin concentrations are communicated via local, auxin-induced, cell-cell signals. We have started to investigate whether cell-cell interactions influence PIN1 polarity by examining the effects of laser-assisted cell ablations. We find that ablation of single cells induces dramatic reorientation of PIN1 in neighboring cells, strongly supporting the hypothesis that local signals coordinate PIN1 polarity patterns. To investigate this phenomenon further we are testing whether different components of the auxin-signaling pathway act autonomously or non-cell autonomously to influence PIN1 subcellular localization.

382. Investigation of PINOID function in the shoot meristem

Marcus G. Heisler, Carolyn Ohno, Zachary Nimchuk

PINOID (PID) is a serine-threonine kinase that has been proposed to act as a binary switch that acts to promote the apical or basal localization of PIN1 depending on PID expression levels. However, although these data imply that the expression of PID is crucial in determining the wt patterns of PIN1 polarity, we still do not yet understand which aspects of the wt localization patterns of PIN1 depend on PID. To investigate this question we have co-visualized PID and PIN1 using different colored GFPs. We find that PID is downregulated after PIN1 polarity converges at a position destined to form a primordium. PID is then upregulated in the boundary region surrounding this position. These data imply that although auxin is thought to promote PID expression, auxin localization is also capable of downregulating PID. To test this hypothesis further we are examining how *PID* expression responds to local auxin treatment in both wild-type and *pid* mutant apices using a kinase-dead mutant PID reporter. To test whether PID is required for PIN1 reversal events surrounding primordia we are using transient knock-out and overexpression methods to visualize dynamically the response of PIN1 to the immediate loss and gain of PID function. Lastly, we are attempting to use FRET to test whether PID directly interacts with PIN1 and if so, where and when this occurs.

383. Chemical approaches to studying the role of the AGC kinase Pinoid in meristem function

Zachary L. Nimchuk, Marcus Heisler, Carolyn Ohno, Elliot M. Meyerowitz

Initiation of lateral primordia is regulated by a pathway involving the hormone auxin. This process is perturbed by mutations in *PID*. *PID* encodes a soluble but membrane-associated AGC family member kinase. Although mutants in *PID* have been studied for a while, little is known about how these pathways regulate patterning of the meristem and there are no identified downstream targets for either pathway. This is partially due to difficulty in interpreting terminal phenotypes in these mutants as well as the presence, either redundant or buffering pathways, which act upon, or are an intrinsic component of the *PID* pathway. In order to circumvent these problems, strategies for live imagining of growing *Arabidopsis* meristems using fluorescent-based markers has been developed by the Meyerowitz lab. Our work is designed to complement these approaches by using chemical genetics to create specifically inhibitible versions of PID. We have made a PID fusion protein with a C-terminus YFP. This protein fusion is detectable in the *PID* proper expression domain and complements the *pid 6* null mutant. Using this construct as a template we have made kinase-dead versions of PID that fail to complement and a dominant negative version of PID that interferes with the wild-type PID function. We are currently testing inhibitable versions of PID for their ability to complement *pid 6* and their responsiveness to specific chemical inhibitors. It is envisioned that these engineered versions will allow for a more in-depth analysis of the genetics and cell biology of these pathways, as well as aide in the identification of pathway targets.

384. Global controls over PIN1 localization patterns and the establishment of organ polarity

Marcus G Heisler, Carolyn Ohno

Previously we have shown that one of the earliest markers of organ polarity, *REVOLUTA*, is expressed adaxially from inception during the early stages of organ formation. This raises the question of how *REV* is prevented from being expressed in abaxial cells at this early stage. To investigate this question we have visualized the expression of the *KANADI1* gene using a functional KAN1-GFP fusion protein. We find that KAN1 is expressed in the periphery of the meristem in a continuous domain that marks the outer boundary of where the PIN1 polarity pattern converges to specify new primordia. Visualization of PIN1, REV and KAN1 simultaneously confirms that KAN1 marks a prepattern correlating with both organ position and the abaxial boundary of REV expression. These observations imply that organ polarity is specified through the positioning of organs on a preexisting boundary of KAN activity such that organ positioning and polarity are inextricably linked. Functional testing of this hypothesis is underway.

385. *Arabidopsis* as a model system for complex regeneration

Sean Gordon

Most multi-cellular organisms have a capacity to regenerate lost features, however, few have the ability to regenerate an entirely new body plan from differentiated tissue. Induction of new shoot apical meristems (SAMs) from cultured root explants is a widely used but poorly understood plant regeneration system in which new plants are regenerated from adult somatic tissue. We have characterized early patterning events during regeneration of the *Arabidopsis* SAM using fluorescent reporters of gene and protein activity known to be required for proper embryonic development. We have found that the early patterning of the new SAM can be broken down into stereotypic stages of *PIN FORMED1* (*PIN1*), *SHOOTMERISTEMLESS* (*STM*), *WUSCHEL (WUS)*, *CUP SHAPED COTYLEDON 2 (CUC2)*, *FILAMENTOUS (FIL)*, *REVOLUTA (REV),* and *CLAVATA3 (CLV3)* expression and activity. We have discovered that new shoot meristems are derived from small number of initial cells via a patterning process in which relative temporal gene expression is analogous to embryogenesis, in contrast to spatial gene expression, which strongly deviates from embryonic patterning. We are further characterizing the shoot regeneration system to support its use as an assay for studying specific aspects of early developmental patterning and an accessible system for the investigation of complex regeneration in the well-studied model organism *Arabidopsis.*

In addition, exogenous hormonal cues dictate the ability of *Arabidopsis* to regenerate. Therefore, we are also investigating the requirement of proper hormone transport in the regeneration process, using both mutants with impaired hormone transport and chemical inhibitors of hormone transport.

386. Retention of molecular memory during regeneration

Sean Gordon

Plants can be typically divided into upper and lower halves corresponding to root, or alternatively, shoot tissue. Through tissue culture and hormonal induction these two fundamentally different tissue types can be converted from one into the other. For example, somatic root tissue can be induced to "de-differentiate" and assemble a new body axis in the form of a shoot meristem. By definition, shoot meristems give rise to all above-ground organs of the plant, including leaves during the vegetative growth phase, followed by flower organs during the reproductive phase. Gene expression, which signals the switch from vegetative growth to the reproductive phase, is known to be systemic (Takada, 2003). We have made the preliminary observation that regenerated shoot meristems resume growth after assembly reflecting the phase of the plant from which the tissue was derived. For example, root tissue derived from plants in the vegetative growth phase regenerate shoot meristems that initiate vegetative shoot growth. On the other hand, root tissue derived from reproductive-stage plants regenerates shoot meristems that quickly undergo reproductive shoot growth. This data conflicts with the current concept of this regeneration system in which cultured somatic root tissue is thought to de-differentiate before re-differentiating into a shoot fate. Instead, our data suggests that a large degree of molecular memory is retained from the original explant tissue. This is consistent with our observation that root-specific genes continue to be broadly expressed during the regeneration process. We are investigating at what level memory of the prior reproductive phase is retained by using a marker of genes known to be involved in the switch between vegetative and reproductive growth.

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387. Function and regulation of *CLAVATA-WUSCHEL* feedback network in SAM patterning and growth

G. Venugopala Reddy

The shoot apical meristem (SAM) is an interacting network of functionally distinct domains. The central zone (CZ) is at the tip of the SAM and harbors a set of stem cells. The peripheral zone (PZ), located on the sides, and the rib-meristem (RM), located just below the CZ, are the regions where cells enter into differentiation pathways to make lateral organs and the stem. SAM size and shape is maintained, though the cells are being continually diverted to differentiation pathways. Intercellular signaling between the CZ and the RM, mediated by *CLAVATA WUSCHEL* genes, has been proposed to function in maintaining the CZ:PZ ratio and also the overall SAM size. However, the mechanisms by which CLV signaling mediates this function are not well understood.

Our work combines transient gene silencing and live imaging approaches to gain mechanistic insights into the process of SAM maintenance. Conditional silencing of the *CLV3* gene was achieved through a dexamethasone (DEX) inducible two-component system. The effect of transient removal of CLV3 function on CZ size was monitored by using a CZ reporter, pCLV3::mGFP5-ER, construct. Expansion of the CZ was mapped with respect to the overall cell division activity within the SAM. This analysis has led to several new insights: 1) CZ expansion occurs through a re-specification process wherein, the existing PZ cells acquire the CZ identity and this process can be uncoupled from growth; and, 2) Overall SAM size is controlled through a process that restricts cell division rates in cells located at a certain distance from the meristem center.

Currently we are extending this analysis to study the regulation of WUS expression and function in SAM maintenance. WUSCHEL (WUS), a homeodomain transcription factor, has been shown to be a critical regulator of SAM maintenance. It has been proposed that CLV signaling controls CZ size and the overall SAM size by negatively regulating the expression of *WUS*. But this proposal is based on observations made during the inflorescence meristem stage, which represents a terminal phenotype. Several hypotheses are possible for the expanded WUS domain in terminal meristems of clv mutants: 1) If *WUS* is negatively regulated by the CLV signaling network, it is expected that the *WUS* domain should enlarge within the RM and/or it should be ectopically expressed in overlying layers; 2) The RM/*WUS* expressing cells could over-proliferate; and, 3) The expanded *WUS* domain in *clv* mutants could be a reflection of altered cell types within the RM. These hypotheses can best be tested in real-time experiments by monitoring the expression of *WUS*, upon compromising or hyper-activating CLV signaling. The required fluorescent reporter construct, pWUS::mGFP5-ER, has been generated and it is being combined with the inducible *CLV3* RNAi system and also the inducible *CLV3* overexpression system.

We are also utilizing the inducible perturbation of CLV signaling to study the interactions between cells in the CZ and the PZ. This analysis would involve expression analysis of markers expressed in the PZ, upon transient perturbations in CLV signaling.

388. Cell biological, genetic and chemical approaches to studying the Clavata1 receptor kinase

Zachary L. Nimchuk, Venugopala Reddy, Xiang Qu, Elliot M. Meyerowitz

Proliferation of cells in plants is restricted to specific regions called meristems. These regions give rise to all the above-ground organs of plants (shoot and floral meristems) and below-ground organs (root meristems). Studies have shown that shoot meristems are organized into functionally distinct cell populations: the central zone (CZ), peripheral zone (PZ), and the rib zone (RZ). The CZ is composed of stem cells that divide slowly relative to cells in the flanking PZ. The RZ subtends the CZ and gives rise to the ground tissues of the stem. In addition, the shoot and floral meristems also give rise to flanking primordia that develop into various aerial organs. This process continues throughout the life of the plant and contributes to overall growth and body plan development. Meristems must balance the number of generated and departed cells in order to maintain themselves. A component of this process includes the regulation of proper spatial initiation of primordial outgrowth on meristem flanks. This process of balanced proliferation and patterning is strictly controlled and is defined genetically by *Arabidopsis* mutants affecting meristem regulation and formation. Mutations in the *CLV* loci lead to a hyper-accumulation of stem cells in both shoot and floral meristems resulting in stem fasciation, club-shaped siliques and extra floral organs. *CLV1* encodes a receptor-like kinase (RLK) with extracellular leucine-rich repeats and a cytoplasmic serine-threonine (ser-thr) signaling domain that may interact with the putative peptide ligand CLV3. We have made a CLV1 fusion protein construct containing a C-terminal YFP tag that fully complements the *clv1 6* mutation. When expressed from the endogenous *CLV1* promoter, CLV1 expression is observed in the central domain of inflorescence and floral meristems. CLV1 appears to localize to the PM and internal structures. We are currently exploring the relationship between localization of *CLV1* and *CLV3* function. We are also exploring this relationship in the context of kinase dead versions of CLV1 and dominant negative CLV1 mutants containing substitutions in the external LRR domain. In addition we are developing a chemical genetic approach using specifically inhibitable versions of the CLV1 kinase. It is envisioned that these engineered version, combined with cell biology, biochemistry and genetic approaches, will allow for a more in-depth analysis of the regulation of CLV1 and potentially aide in the identification of pathway targets.

389. Cell fate decision by CLV in shoot apical meristem

Xiang Qu, G. Venugopala Reddy

Located at the growing tip of stems, shoot apical meristems (SAMs) are actively dividing, embryonic tissues responsible for all of the aerial organs in plants. A balance between meristematic cell division and differentiation is required to maintain a functional SAM. In *Arabidopsis*, the *CLAVATA* (*CLV*) genes encode important elements in SAM maintenance. Single loss-of-function mutations of the *CLV* genes (*clv*) result in a progressive enlargement of shoot and floral meristems. *CLV1* encodes a transmembrane protein that belongs to a large family of receptor-like kinases (RLKs) in plants. *CLV3* encodes a small extracellular protein with a putative cleavage signal. Genetic evidence indicates that the CLV genes act in concert to restrict the size of the SAM. In this hypothetical system, CLV1 acts as a plasma membrane-bound receptor. The signal perceived from the intercellular CLV3 ligand

will be transduced to the downstream signaling component, theoretically through the C-terminal kinase domain of the CLV1 receptor. However, no direct biochemical or molecular evidence supports this model. We have been using a combination of methodologies, including biochemical, genetic, and cell biological approaches, to gain information in molecular detail as to how the receptor-like kinase CLV1 functions as a key element to maintain a functional SAM. We have generated constructs that allow us to visualize the CLV proteins directly using a confocal microscope and/or detect them indirectly by chemi-luminescence. We have demonstrated that CLV1 is localized to the plasma membrane. An analysis of interaction between CLV1 and CLV3 and its biological significance has been undertaken. Results from the study of CLV3, potentially leads to the discovery of a novel mechanism by which the SAM is maintained. In addition, to address whether the kinase domain of CLV1 and/or the associated enzymatic activity are required for CLV signaling, we have made several constructs containing individual mutations which would result in abolition of the kinase activity of CLV1. The analysis of physiological output of those constructs is currently under way.

390. Expression analysis of the CLE family of putative secreted ligands in *Arabidopsis*

Adrienne Roeder, Nicole Kubat

Plant and animal cells must communicate with one another to coordinate their actions during development. One mechanism, among many, that cells use to communicate is the secretion of small signaling peptides that diffuse to the surrounding cells. The receiving cells sense the presence of the signaling peptide through receptors on the cell surface and respond accordingly. In the small plant *Arabidopsis*, such a signaling pathway is used in a feedback loop to maintain the size of the stem cell population at the growing tip of the plant, or meristem. The *CLAVATA3* (*CLV3*) gene encodes a small, secreted ligand that is expressed in the stem cells and signals through the *CLAVATA1* (*CLV1*) leucine-rich repeat receptor-like kinase to downregulate expression of the transcription factor *WUSCHEL* (*WUS*) in the underlying cells (Clark *et al*., 1997; Fletcher *et al*., 1999; Schoof *et al*., 2000; Brand *et al*., 2000; Rojo *et al*., 2002). *WUS* by an unknown mechanism feeds back to positively regulate *CLV3,* thus maintaining the constant size of the meristem. In *clv3* mutants where this balance is disrupted the meristems becomes greatly enlarged, whereas if *CLV3* is constitutively expressed all of the stem cells are consumed and the meristem terminates (Clark *et al*., 1995; Brand *et al*., 2000).

CLV3 was the founding member of a family of putative small, secreted ligands termed the CLE family (Cock and McCormick, 2001). To uncover the roles of the other *CLE* genes, we are undertaking a multi-pronged high-throughput analysis of all 25 of the *Arabidopsis* CLE family members in a large collaborative effort with Jennifer Fletcher's lab at the Plant Gene Expression Center and Lewis Feldman's lab at U.C. Berkeley (http://www.pgec.usda.gov/Fletcher/CLEproject.html).

Although initially it appeared that many of the *CLE* genes were expressed broadly based on RT-PCR data from different tissues (Sharma *et al*., 2003), we have generated reporter constructs for *CLE9*, *CLE10*, and *CLE22* and found that each of these genes has a specific expression pattern during plant development. Our strategy will be to match the expression pattern to that of putative receptors whose ligands are unknown.

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391. Dynamic analysis of the GATA-like transcription factor HANABA TARANU during *Arabidopsis* development

Xing Qu, Yuanxiang Zhao

HAN (*HANABA TARANU*) encodes a GATA-like transcription factor and is essential for floral development in *Arabidopsis*. All four identified *han* mutants (*han 1*, *han 2*, *han 3*, and *han 4*) display dramatic floral phenotypes, with fused sepals and reduced organ number throughout all four floral whorls. The expression pattern of *HAN* in the shoot apical meristem (SAM) and floral meristem is distinctive, with strong expression at the boundaries between the meristem and its newly initiated organ primordia, and at the boundaries between different floral whorls. Although *han* mutations have minor effects upon vegetative SAMs, *han;clv* double mutants display a highly fasciated SAM. Together along with the observation that the WUS expression pattern is altered in *han* mutants, our data suggest that HAN is also involved in SAM development. To understand the molecular mechanism by which HAN regulates flower and SAM development, in collaboration with Dr. Wolfgang Lukowitz at Cold Spring Harbor Labs, we have conducted a second-site screen for suppressors from the EMS-mutagenized *han 2* plants. About 20,000 mutagenized plants from 40 pools were screened, among which 64 repressors and 25 enhancers were identified. Further analysis and mapping of these mutants is currently underway. As an alternative approach, we have developed a hormone-inducible system that allows us to activate HAN upon the treatment of plants with dexamethasone (DEX). Following the activation of HAN, we can monitor changes in the expression patterns of selected floral and SAM genes (*WUS*, for example) in live imaging. Using the same system, we are planning to conduct microarray analysis in a time course after induction. The time course should be quite useful in indicating downstream players, and for correlating gene expression patterns with

morphogenic events. The immediate targets from the microarray experiments will be selected for chromatin immunoprecipitation.

392. Analysis of MOB protein localization

Carolyn Ohno

The *MODIFIER of B Function (MOB)* gene encodes a putative transcription factor that negatively regulates the expression of the petal and stamen organ identity gene *PISTILLATA (PI)*. *mob* mutations suppress the *ufo* floral phenotype associated with reduced petal and stamen development. UFO encodes an F box-containing factor thought to act as an SCF-type E3 ubiquitin ligase. One hypothesis for UFO function is that it targets a repressor of *PI* expression for proteolysis. Since *mob* is epistatic to *ufo*, it is possible that MOB is the repressor targeted by UFO. To test this possibility we have examined the expression of a pMOB::MOB-CFP translational fusion protein and we find that it is nuclear-localized, ubiquitously-expressed and functional in that it can rescue the *mob* mutant phenotype. This is consistent with the ubiquitous *MOB* mRNA expression pattern detected by *in situ* hybridization. Confocal imaging of living meristems and flowers harboring a functional pUFO::UFO-VENUS translational fusion protein that is spatially restricted to the peripheral region within the meristem and to petal and stamen primordia in floral buds, indicates that the presence of UFO protein does not influence the uniform accumulation of MOB-CFP within the inflorescence. We have also examined MOB-CFP protein accumulation in *ufo* mutant or *35S::UFO* genotypes and find that it is unchanged. Thus, our evidence suggests that MOB and UFO act in parallel pathways in which the repression of *PI* by MOB does not require UFO activity.

393. Giant cells in the sepal epidermis of *Arabidopsis* flowers

Adrienne Roeder

Although the homeotic genes that control floral organ identity in *Arabidopsis* have been known for more than a decade, we still know little about how they control the downstream processes of cell division, cell expansion, and cell fate specification to generate complex floral organs composed of many difference cell types. The sepals, the external floral organs that surround and protect the developing bud, are a good system for addressing this question because the outer epidermis of the sepal contains extremely enlarged giant cells interspersed in the smaller epidermal cells (along with trichomes and guard cells). The giant cells average 364 μm (±145 s.d.) in length, but can stretch almost half the length of the mature sepal. One of the initial questions to address is whether the giant cells are truly a developmentally distinct cell type or are merely larger cells in a continuum of epidermal cell sizes. In floral buds, the distinction between the sizes of the giant cells and small cells is clear. The average area of the giant cells is 2600 μm^2 (±640) whereas the small epidermal cells average only 88 μm^2 (±74). In addition, two molecular markers, one expressed specifically in the giant cells and one expressed specifically in the smaller epidermal cells, suggest that these cell types are have different patterns of gene expression. Finally, the giant cells contain enlarged nuclei, suggesting that they have undergone endoreduplication, a specialized cell cycle in which the DNA is replicated, but the cell does not divide. To identify genes involved in giant cell development, we have been conducting a screen for mutants that affect giant cell formation. We have isolated a mutant in which giant cell formation is deficient and a mutant in which all of the sepal epidermal cells appear enlarged similar to giant cells.

394 Analysis of the gene regulatory network underlying early *Arabidopsis* flower development

Frank Wellmer, Elliot M. Meyerowitz

Flower development is an excellent model system for studying organogenesis in plants. Extensive genetic analyses, especially in the model plant *Arabidopsis thaliana* have led to the identification of several key regulatory genes of this important biological process. The vast majority of these genes encode transcription factors or other proteins involved in the regulation of gene expression, indicating the existence of a complex gene regulatory network that underlies flower development. We are interested in understanding the architecture and composition of this gene network at a molecular level.

To identify genes involved in flower development on a genome-wide scale, we have analyzed the global gene expression dynamics in early flower development, a key process in the life cycle of a plant, during which floral patterning and the specification of floral organs is established. We used a novel floral induction system, which allows the isolation of a large number of synchronized floral buds, in conjunction with whole-genome microarray analysis to identify genes with differential expression at distinct stages of flower development. We found that the onset of flower formation is characterized by a massive downregulation of genes in incipient floral primordia, which is followed by a predominance of gene activation during the differentiation of floral organs. Among the genes we identified as differentially expressed in the experiment, we detected a significant enrichment of closely related members of gene families. The expression profiles of these related genes were often highly correlated, indicating similar temporal expression patterns. Moreover, we found that the majority of these genes is specifically up-regulated during certain developmental stages. Because co-expressed members of gene families in *Arabidopsis* frequently act in a redundant manner, these results suggest a high degree of functional redundancy during early flower development, but also that its extent may vary in a stage-specific manner.

We are now using the genome-wide information on gene expression obtained in this study, as well as the floral induction system to systematically study the gene regulatory network underlying flower development.

395. The homeotic protein AGAMOUS controls late stamen development

Toshiro Ito[*]*, Hao Yu*[*]

The floral homeotic selector gene *AGAMOUS* (*AG*), together with other members of the same class of floral homeotic genes, is sufficient to trigger reproductive organ (stamen and carpel) development in the context of a developing flower. Although *AG* is the most studied example of a plant homeotic gene, the mechanisms by which a floral primordium responds to the genetic activities initiated by AG, and thereby develops stamens and carpels, are largely unknown. *AG* RNA is expressed in the organ primordia of floral whorls 3 and 4 (the positions of stamens and carpels, respectively) from floral stage 3 until late in flower development, after all of the organs are fully formed. While early *AG* expression acts in specification of stamens and carpels, the role, if any, of continued *AG* expression in later flower development is unknown.

In order to examine the timing of AG action and its possible late stage functions, we performed a series of time course experiments using a transgenic line with inducible AG activity, in an *ag* homozygous mutant background. We showed that ectopic AG activity can transform organ primordia of as late as stage 6 floral buds into normal-looking stamens. In contrast, activation of AG in floral buds after stage 7 results in 2nd and 3rd whorl organs that are stamen-like filamentous petals. This suggests that AG can induce the gene activities necessary for filament elongation, but not for microsporogenesis, at late floral stages. We also show by using timed activation of AG that AG controls late-stage stamen development, including anther morphogenesis and dehiscence, as well as filament formation and elongation. This demonstrates that prolonged AG activity is necessary to form wild-type stamens in *Arabidopsis*. Our results suggest that stamen identity and differentiation control by AG is achieved by the regulation of different transcriptional cascades in different floral stages, with organ specification cascades induced early, followed by activities necessary for filament formation and anther maturation.

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396. Genetic redundancy and subfunctionalization among plant microRNAs: Role of the *miR164* family in developmental homeostasis

Patrick Sieber[*]*, Frank Wellmer, José Luis Riechmann*

MicroRNAs (miRNAs) are short single-strand RNA molecules that control plant and animal gene expression. Although miRNAs have been studied extensively in recent years, few loss-of-function studies have been reported. In plants this might be because many miRNAs are represented by more than one locus and loss of an individual miRNA might be compensated for by an other member of the same family. Here it is shown that all members of the *miR164* miRNA family of *Arabidopsis thaliana* are functionally redundant during shoot development. *miR164c*, in addition, has some unique functions indicating subfunctionalization of individual *miR164* miRNAs. Elimination of the activity of all members of the *MIR164* miRNA family leads to a randomization of certain developmental patterns in *Arabidopsis thaliana*, revealing a role for *miR164* miRNAs as genetic suppressors of phenotypic variation. Molecular and genetic dissection of *miR164* mediated target regulation indicates that all *miR164* miRNAs function together to control the level of target gene abundance and to prevent fluctuations in the expression of their target genes and to prevent variability in organ growth, organ number, organ size and organ position during shoot development. Our results indicate a negative correlation between high abundance of the *miR164* targets *CUP SHAPED COTYLEDON (CUC)* and growth. Candidate genes with a potential role in cell elongation and cell division rates and which might be regulated by CUC1 and CUC2 were identified in a transcriptome analysis of plants with altered *CUC* transcript abundance by using our whole-genome *Arabidopsis* microarray.

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397. Characterization of the *Arabidopsis* mutant *SNOWBALL*

Zachary L. Nimchuk, Elliot M. Meyerowitz

We have isolated an apparently novel mutant which we have named *SNOWBALL*. *SNO* mutants display various defects indicative of altered meristem function. *SNO* mutants display alterations in floral organ number, with the most prominent effects being an increase in sepal number and a reduction in fertility. All vegetative and floral organs of *SNO* mutants are initially thinner than wild type, however, leaves expand to full size but are frequently twisted. In addition, floral organ phyllotaxis is altered. *SNO* mutants frequently display ectopic outgrowths, similar to mutations in the *REVOLUTA* gene. The *SNO* mutant is not allelic to *REV* and *SNO* mutants do not display other *REV* phenotypes such as defects in polarity or auxiliary meristem development. We are currently mapping *sno* and examining *sno* interactions with various mutants.

398. Identifying transcriptional targets of leaf growth

Carolyn Ohno

To understand growth and morphogenesis of plant lateral organs, we have continued characterization of JAGGED (JAG), a putative zinc finger transcription factor that is expressed in leaf and floral organ primordia. Loss of *JAG* function results in the development of narrow jagged leaves and floral organs due to defects in both cell divisions and cell expansion. In contrast, misexpression of *JAG* in the floral meristem promotes the development of leaf-like tissue in place of flowers, suggesting that *JAG* is sufficient to specify leaf growth. Whole genome oligonucleotide microarrays are being used to identify

downstream transcriptional target genes of *JAG* that may be involved in specifying leaf cell fate or cell proliferation. Transgenic lines that express dexamethasone (DEX) hormone-inducible *JAG* under the control of either a constitutive *CaMV 35S* promoter or the endogenous *JAG* promoter have been generated in the *jag* mutant background and a time course study of gene expression after 7, 9 and 12 hours after DEX induction of *JAG* in seedlings has been analyzed with replicates. At 7 hours after *JAG* induction, 88 genes are upregulated while 49 genes are downregulated greater than two-fold (p-value < 0.001). Included among the changed *JAG* target genes are transcription factors and hormone regulators and we are currently investigating the RNA expression patterns and loss- and gain-of-function phenotypes of these candidates.

399. MscS-like proteins in plants

Elizabeth S. Haswell

Mechanotransduction is the process by which physical information about the extra- and intracellular environment is converted to a biochemical signal. How plants respond to mechanical stimuli has been under investigation since the work of Darwin [1], but little is known about the molecules involved. Response to mechanical stimuli such as gravity, temperature, turgor pressure, and touch are important for plant growth and development. Tension-responsive ion channel activities have been discovered in the plasma and vacuolar membranes of many plant species and plant cell types. However, the molecular identities of these channel activities are not known, nor have they been clearly correlated with a physiological function. Candidates for the proteins underlying these activities in plants are MscS-like proteins. There are ten MscS-Like (MSL) family members in *Arabidopsis thaliana*, which can be divided into two classes. Class I proteins are the most similar to MscS and its bacterial relatives, and GFP-fusion proteins are found in the mitochondrial or chloroplast envelopes. Class II proteins are most similar to other MscS-like proteins in plants, and are localized to the tonoplast and the plasma membrane. MSLp::GUS reporter genes exhibit tissue-specific expression, most notably in guard cells, the vasculature, and the stigma cells of the carpel. This initial characterization of the MSL proteins lays the foundation for future experiments designed to evaluate their role as mechanoreceptors in plants.

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400. MscS-like proteins control plastid size and shape in *Arabidopsis thaliana*

Elizabeth S. Haswell

Mechanosensitive (MS) ion channels provide a mechanism for the perception of mechanical stimuli such as sound, touch, and osmotic pressure. The bacterial MS ion channel MscS opens in response to increased membrane tension and serves to protect against cellular lysis during osmotic downshock. MscS-like proteins are found widely in bacterial and archaeal species, and have also been identified in fission yeast and plants. None of the eukaryotic members of the family have yet been characterized. We characterized two MscS-Like (MSL) proteins from *Arabidopsis thaliana*, MSL2 and MSL3 [1]. MSL3 can rescue the osmotic shock sensitivity of a bacterial mutant lacking MS ion channel activity, suggesting that it functions as a mechanosensitive ion channel. *Arabidopsis* plants harboring insertional mutations in both *MSL3* and *MSL2* show abnormalities in the size and shape of plastids, plant-specific endosymbiotic organelles responsible for photosynthesis, gravity perception, and numerous metabolic reactions. MSL2-GFP and MSL3-GFP are localized to discrete foci on the plastid envelope, and co-localize with the plastid division protein AtMinE. Our data support a model wherein MSL2 and MSL3 control plastid size, shape, and perhaps division during normal plant development by altering ion flux in response to changes in membrane tension. We propose that MscS family members have evolved new roles in plants since the endosymbiotic event that gave rise to plastids.

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401. Functional analysis of the *msl2-1* and *msl3-1* mutant alleles

*Elizabeth S. Haswell, Susana Nava**

The ten MscS-Like (MSL) proteins of *Arabidopsis* show sequence similarity to the bacterial mechanosensitive ion channel MscS. MscS functions as an osmotic safety valve, allowing the non-selective release of ions upon extreme hypoosmotic shock [1]. To determine the function of two of these MSL proteins, MSL2 and MSL3, insertional alleles were isolated from pools provided by the University of Wisconsin. In both *msl2 1* and *msl3 1* alleles, the T-DNA insertion is located in the last exon of the gene, downstream of the transmembrane domains and MscS-related domain. As the levels of *MSL2* and *MSL3* mRNA are normal in *msl2 1* and *msl3 1* mutants, we are taking several approaches to investigate the nature of the defect in these mutants. First, we are testing the phenotypic effect of perturbing *MSL2* and *MSL3* expression in various ways. For example, we are in the process of obtaining an allele of *MSL2* with an insertion in the first exon. We have also reduced *MSL3* levels through RNA interference; in a *msl2 1* background this results in variegated leaves and enlarged chloroplasts, phenocopying the *msl2 1; msl3 1* double mutant [2]. In another approach, we are characterizing the truncated proteins, MSL2t and MLS3t, which are predicted to be produced in the *msl2*-1 and the *msl3*-1 alleles. Overexpression of MSL3t and MSL2t in a wild-type background produces plants that phenocopy the double *msl2 1; msl3 1* plants, suggesting that *msl2 1* and *msl3 1*

are gain-of-function alleles. Extremely high expression of the full-length GFP-fusion proteins can also produce enlarged chloroplasts. MSL3 is capable of rescuing the osmotic shock sensitivity of an *E. coli* strain that lacks MS ion channel activity; when tested in this assay MSL3t functions normally. MSL2- and MSL3-GFP fusion proteins are localized to foci at the poles of the plastid envelope. We find that MSL2t- and MSL3t-GFP fusion proteins are also targeted to the plastid envelope, but are evenly dispersed around the periphery of the plastid, rather than localized to foci. These data are consistent with a model wherein the *msl2 1* and *msl3 1* mutant alleles produce truncated proteins that can form functional ion channels but are not properly localized; and that localization of the putative MS ion channels MSL2 and MSL3 to polar foci is required to properly control plastid size and shape and to prevent variegation. We are currently more narrowly defining the sequence at the extreme C-terminus of MSL2 and MSL3 that required for localization to foci, and further characterizing the function of MSL2t and MSL3t in *in vitro* interaction and stability assays.

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402. The role of MSL9 and MSL10 in the osmotic shock response of the *Arabidopsis* root

Elizabeth S. Haswell, Remi Peyronnet[*], Helene Barbier Brygoo[*]

MSL9 and MSL10 are Class II members of the MscS-Like (MSL) protein family in *Arabidopsis*; they are most related to other plant MscS-like proteins, contain six putative transmembrane domains, and do not contain organelle transit sequences. MscS is a bacterial mechanosensitive (MS) ion channel that functions as a safety valve, allowing ions to exit the cell and preventing plasmolysis in response to extreme hypoosmotic shock [1]. We therefore hypothesized that MSL9 and MSL10 might function in the osmotic shock response of the *Arabidopsis* plant. Treatment of whole seedlings with 0.44 M mannitol or 100 μM abscisic acid, two treatments known to induce osmotic shock or dehydration [2], induced the expression of MSL9 and MSL10. RT-PCR analysis demonstrated that MSL10 is widely expressed throughout the plant, while MSL9 expression is primarily expressed in the tip of the root. GUS reporter lines verify these data and further refine the expression patterns, showing complementary expression patterns for the two genes. MSL10p::GUS is expressed strongly in the vasculature of the root and shoot, while MSL9p::GUS activity is found in the columella cells of the root cap and the cortex and endodermal cell layers of the root elongation zone. Low levels of GUS activity are also found at the base of young leaves, but appears to be excluded from the vasculature. As they are related to a mechanosensitive ion channel, MSL9 and MSL10 may directly respond to changes in membrane tension that result from osmotic stress. A stretch-activated ion channel activity has been characterized in root protoplasts using a cell-attached patch clamp method. This activity is increased by negative pressure in the patch pipette (suction) and is inhibited by treatment with 1mM gadolinium ion, an inhibitor of MS ion channel activities. Preliminary data suggests that this activity is absent or reduced in *msl9 1; msl10 1* double-mutant plants. We are currently verifying these results and preparing to study the activity under question in excised patches. Further, we are determining the subcellular localization of MSL9- and MSL10-GFP fusion proteins.

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403. Do MSL7 and MSL8 play a role in pollen hydration?

Elizabeth Haswell, Arnavaz Garda

MSL7 and MSL8 are two members of the MscS-Like (MSL) protein family in *Arabidopsis*. These two genes are 71% identical at the amino acid level, and are located in tandem on chromosome two. To determine the function of these genes, we have begun to characterize their expression pattern, subcellular localization, and to identify insertional mutant alleles. *MSL8* expression was not detected by use of GUS reporter lines or by RT-PCR. However, MSL7p::GUS reporter lines show a distinctive expression pattern, with GUS activity restricted to the stigma cells of the carpel. RT-PCR data verifies that *MSL7* transcripts are found only in the flower. The stigma cells of the carpel are important for interaction with and hydration of the pollen grain before pollen tube growth and fertilization can begin [1]. Specific expression in stigma cells, coupled with the putative function of these proteins as mechanosensitive ion channels, suggests that MSL7 and perhaps MSL8 function in the process of pollen hydration. To test this hypothesis, we identified insertional alleles in both *MSL7* and *MSL8*. However, *msl7 2* and *msl8 4* homozygous plants appear to have wild-type structure and function of stigma cells. As it is likely that these two closely related genes function redundantly, we are working towards generating a *msl7; msl8* double mutant. To do this, a transposon located in the first exon of *MSL8* has been mobilized, and we are currently preparing to screen F2 plants for insertion into the nearby *MSL7* gene. We are taking a second approach to perturbing MSL7 and MSL8 function, by generating a RNA-interference construct that will reduce the levels of both *MSL7* and *MSL8* transcripts.

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404. A genetic screen for enhancers of *msl2-1*

Elizabeth S. Haswell

The *Arabidopsis MSL2* and *MSL3* genes encode putative mechanosensitive (MS) ion channels related to the bacterial MS ion channel MscS. No phenotype has yet been detected in *msl2 1* or *msl3 1* mutant plants, but *msl2 1; msl3 1* double mutants have variegated leaves, enlarged chloroplasts, and enlarged, spherical epidermal plastids [1]. Variegation, or the presence of differently colored tissues in the leaf, has been observed in a number of mutants and is frequently attributed to defects in plastid development [2]. It has been proposed that a plastid-to-nucleus signal prevents proper leaf development when plastid development is disrupted. The *msl2 1; msl3 1* mutant is unusual in that it is variegated but chloroplasts are normally developed, through greatly enlarged. Other mutants with enlarged plastids, such as the *arc (accumulation and replication of chloroplasts)* mutants also have enlarged plastids, but none of them exhibit variegation [3]. To begin to understand the nature of the variegation pattern observed in the *msl2 1; msl3 1* double mutant, we have initiated a genetic screen for variegated mutants in the *msl2 1* single-mutant background. 20,000 seed were treated with 0.4% of EMS for 8 hours, washed thoroughly, and planted. M2 seed was collected in pools of 40 and 160 seed from each of 10 pool planted. Seed from 56 mutant plants with visible variegation was collected. We are now verifying these phenotypes in the M3, performing backcrosses and complementation tests, and generating mapping populations. Mutants that phenocopy the *msl2 1; msl3 1* mutant in that they have enlarged chloroplasts are likely contain new alleles of *MSL3*, or genes that control MSL2 or MSL3 function. Mutants that are variegated but do not have enlarged chloroplasts may contain lesions in genes downstream of *MSL2* in the putative plastid-to-nucleus signal transduction pathway. We are interested in both classes of mutants, and preliminary analysis of the chloroplast phenotype of several M2 lines indicates that both classes are present in our mutant collection.

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Summary: The Rothenberg group studies the molecular mechanisms that are responsible for developmental lineage choice as hematopoietic stem cells differentiate into T lymphocytes. This is a complex process in which stem-cell multipotentiality is lost in steps that actually overlap with the initiation of T-cell specific differentiation events. Thus, it offers unique insights into the nature of "stem-ness" and the distinction between activation of a development program and irreversible commitment to that program. The approaches used in the lab are a combination of *in vitro* developmental biology, high-resolution characterization of individual cell developmental states, and molecular genetics of gene regulation. We focus on identifying the transcription factors and signaling events that induce T-lineage gene expression in an uncommitted precursor and determining how they work to force the cell to relinquish other developmental options. Kinetic dissection of this complex process using *in vitro* differentiation systems and retroviral perturbation make it possible to solve the roles of individual regulatory molecules in successive, highly defined developmental contexts. In addition, the group is investigating the subtle variations in this pathway that may predispose to autoimmunity, and the evolutionary origins of both the T- and B-lymphocyte developmental programs by comparative analysis of basal vertebrates.

Initiation of T-cell development depends on Notch/Delta signaling, and beyond that, it also requires the staged upregulation of a variety of other transcription factors: GATA-3, TCF-1, probably Bcl11b, and specific forms of the bHLH transcription factor HEB. One set of projects in the lab aims to reveal exactly how each of these regulatory molecules contributes to the onset of T-cell gene expression. Even as these T lineage-associated factors are being induced, the cells preserve a stem cell-like pluripotency through many cell divisions into the beginning of their T-cell program. Throughout this period, the cells also continue to express a surprisingly broad range of regulatory factors inherited from hematopoietic stem cells. What eventually "locks down" the T cell identity in these cells, then, appears to be a separate mechanism dominated by repression: Repression of regulatory genes like PU.1, which play positive roles in stem cells before T-lineage differentiation but also can actively maintain access to non-T developmental options. In a second major interest of the lab, we are now closing in on the identification of the key repressors that terminate PU.1 expression.

Some take-home lessons have emerged about the nature of T-lineage specification process as a whole. It is not a simple command cascade of dedicated regulators, such as appear to drive differentiation of other blood-cell types. Instead, many if not all of the critical T-lineage regulatory genes are used for other pathways as well and can even push early T-lineage cells to alternative fates under certain circumstances. Their essential roles in T-cell development are completely context-dependent and dose-dependent. This is true not only of factors like PU.1, which plays a transient-positive role in T-cell development, but also of GATA-3, which is used repeatedly throughout T-cell development. Thus, the regulatory network context gives these factors their T-lineage promoting impact, and solving its structure is a major goal of the lab. This year, we have shown that Notch/Delta signaling plays a sustained role in this regulatory context long after it initiates the whole T-lineage differentiation process, transforming the net impact of the other regulatory factors, and protecting the cells from lineage diversion through at least two different mechanisms. Another insight that has emerged is how close in regulatory terms developing T-cell precursors remain to cells of the "innate immune system," such as dendritic cells, macrophages, and mast cells.

Recently, Dr. Mary Yui in our group has shown that the earliest checkpoint that normally provides "quality control" for newly committed T-lineage cells is altered in function in mice that are genetically susceptible to autoimmune diabetes. The checkpoint affected, β-selection, is complex, including a life vs. death threshold and completion of T-cell lineage commitment as well as a less-understood developmental branch point between αβ and γ lineage T cells, which differentiate using somewhat different transcriptional programs. The identification of the checkpoint alteration in autoimmunity-prone mice provides an exciting opportunity because autoimmune diabetes is a multigene disease that has been difficult to dissect into discrete "component phenotypes." Dr. Yui is now leading a project to map the genetic defects that contribute to this alteration and to determine its molecular basis. The goal is to reveal in detail how subtle early developmental defects may result in a mature T-cell population with key failures in self-restraint mechanisms. A physician-scientist in the group, Dr. Chace Tydell, is also pursuing a separate investigation of the innate-immunity contributions to autoimmune disease.

In exploring the evolutionary origins of the complex lymphocyte developmental program, we focus on

the interface between the conventional B and T lymphocyte-based immune systems of jawed vertebrates and the lymphocyte-independent innate immune systems of all invertebrates. The discontinuity between these systems is represented by the lamprey, a jawless vertebrate with lymphocytes that recognize antigen using highly polymorphic receptors, but receptors of a completely different structural type than the B- and T-cell receptors of all jawed vertebrates. The lamprey lymphocytes cannot be clearly identified as more T-like or more B-like, and the animal lacks recognizable thymus in which a specialized T-cell program could be induced through Notch/Notch ligand interaction. Thus, it is of some interest to understand the regulatory signature of the lamprey lymphocytes, as a clue to what the ancestral immune-cell developmental program might have been. To do this, we are starting from the known lamprey lymphocyte receptor genes and using their regulatory sequences to determine what kinds of factors control their expression, in collaboration with Drs. Max Cooper, Chris Amemiya, and Zeev Pancer, and with the Bronner-Fraser lab. This year has brought some exciting progress in the mapping of the key sites, presenting a prospect of understanding this truly ancient family connection in the near future.

405. Notch/Delta signaling modulates PU.1 activities to constrain developmental re-engineering of pro-T cells

Deirdre D. Scripture Adams[1], Christopher B. Franco[1,2], Irina Proekt[3], Tom Taghon, Mary A. Yui, Stephanie L. Adams, Rochelle A. Diamond, Ellen V. Rothenberg

PU.1 is essential for early stages of mouse T-cell development but antagonizes it if expressed constitutively. Two separable mechanisms are involved, attenuation and diversion. Dysregulated PU.1 expression inhibits pro-T cell survival, proliferation, and passage through β-selection by blocking essential T-cell transcription factors, signaling molecules, and *Rag* gene expression, and expression of a rearranged TCR transgene does not rescue. However, Bcl2 transgenic cells are protected from this attenuation and may even undergo β-selection, as shown by PU.1 transduction of defined subsets of Bcl2-transgenic fetal thymocytes with differentiation in OP9-DL1 and OP9-control cultures. The outcome of PU.1 expression in these cells depends on Notch/Delta signaling. PU.1 can efficiently divert thymocytes toward a myeloid-like state with multigene regulatory changes, but Notch/Delta signaling vetoes diversion. Gene expression analysis distinguishes sets of critical T-lineage regulatory genes with different combinatorial responses to PU.1 and Notch/Delta signals, suggesting particular importance for inhibition of E proteins, Myb, and/or Gfi1 in diversion. However, Notch signaling only protects against diversion of cells that have undergone T-lineage specification, after Thy-1 and CD25 upregulation. The results imply that in T-cell precursors, Notch/Delta signaling normally acts to modulate and channel PU.1 transcriptional activities during the stages from T-lineage specification until commitment. We are currently expanding our understanding of PU.1 function through the use of mouse models and *in vitro* knockdown technology.

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406. An essential stage specific role for GATA-3 in early T-cell development

Deirdre D. Scripture Adams

The T regulatory transcription factor GATA-3 is expressed throughout intrathymic T-cell development as well as during peripheral T-cell development (where it has a role in promoting the shift to the T_H2 phenotype). GATA-3 can act as a repressor, an activator, and a chromatin-remodeling factor. Our previous studies suggest that GATA-3 is important in the earliest stages of T-cell development, but an absolute requirement for this factor at each of the earliest stages has not been proven. Each of the early stages in T-cell development is functionally and genetically distinct: They can be divided into T specification, T-lineage commitment, and TCR β-chain selection. Each of these developmental states is achieved through a defined panoply of expressed genes, including many transcription factors. We are now using a variety of gene expression knock down techniques (including retroviral expression of shRNA and electroporation of siRNA or morpholinos specific for GATA-3) to discover whether GATA-3 expression is required in each of the earliest DN stages, either for survival or for developmental progression. In these experiments, we are also identifying target genes regulated by GATA-3, and thus defining the control exerted by this factor over the formation and composition of the gene expression networks controlling T-cell development.

Our work so far has shown a requirement for GATA-3 at the early T precursor stage, as well as at DN1, DN2, and DN3. The developmental blocks observed at the precursor and DN1 stages are the most severe, with most DN1 cells dying in the absence of GATA-3 after only 5 days of *in vitro* culture. At DN2, loss of GATA-3 does impede development, but does not affect viability. In DN3-stage cells, both developmental progression and viability are impaired. None of the blocks at these stages can be overcome by providing a bcl-2 transgene, although decreases in viability can be somewhat relieved at the precursor and DN1 stages using this method. Single cell culture experiments support the importance of GATA-3 for viability and developmental progression, as GATA-3 shRNA expressing clones failed in all cases to progress beyond DN2.

In an initial analysis of gene expression changes that occur in response to the loss of GATA-3, we find that PU.1 (a potent promoter of myeloid fates) as well as Mac-1, CD8α, and CD25 among others, are all upregulated. If PU.1 is directly regulated by GATA-3, this may explain the mechanism of the block in development we have observed, as PU.1 may be promoting a myeloid

fate in these immature T cells when GATA-3 is lost. We are currently conducting this type of gene expression analysis within each stage of T development, and will use this information to understand the precise GATA-3 mechanism in action at each developmental phase.

407. Mast-cell diversion of T-lineage precursor cells by the essential T-lineage transcription factor GATA-3

Tom Taghon, Mary A. Yui*, Stephanie L. Adams, Ellen V. Rothenberg*

GATA-3 is a T-lineage-specific hematopoietic transcription factor that is induced by Notch/Delta signaling and essential for T-cell development from the earliest stages. However, GATA-3 in itself does not instruct T-lineage determination. Its action in lymphoid precursors is profoundly dependent on level, Notch signaling, and developmental stage. Although Notch signaling and GATA-3 at normal levels collaborate to promote T-cell development, and Notch signaling is critical for pro-T cell survival, overexpression of GATA-3 reverses these relationships. High-level GATA-3 blocks T-lineage differentiation and survival in the presence of Notch signals, while conferring a survival advantage in the absence of Notch signals. Furthermore, high-level GATA-3 confers a gain of developmental function. In DN1 and DN2 but not DN3 thymocytes, GATA-3 overexpression induces mast-cell lineage respecification via rapid upregulation of c-Kit, SCL/Tal-1, Mitf, GATA-1, and GATA-2. Effects of the hypomorphic GATA-3-KRR$_{305\text{-}307}$ mutant show that mast cell lineage diversion can be uncoupled from GATA-3 inhibitory effects on pro-T cell survival. The unexpected ease of lineage conversion suggests close relationships between the pro-T and mast-cell precursor regulatory programs and a new role for Notch signaling in restricting non-T cell development in the thymus.

**Equal author contribution*

408. Genome-wide search for GATA-3 target genes using ChIP-chip assay

Jingli Zhang

Within the hematopoietic system, expression of the zinc-finger transcription factor GATA-3 is restricted to thymocytes and T cells. Starting from pre-thymic progenitors, this factor is involved in multiple stages of T-cell development progression and critical for generation of mature T cells. GATA-3 knockout mice die between embryonic day 11 and 12, but study of gene-deficient cells in a blastocyst complementation system has shown that GATA-3-deficient ES cells specifically fail to give rise to thymocytes or T cells. Conditional knockout experiments have implied that it is needed for the DN3/DN4 transition (β-selection) and development of CD4 SP cells (positive selection). It also plays a role in blocking inappropriate development of CD8 SP cells. In peripheral T cells, GATA-3 promotes CD4 T cell differentiation to the T_H2 effector type while inhibiting T_H1 cell differentiation. Several T-cell-specific genes such as RAG-2, TCR gene enhancers, CD8 and IL-4 have been suggested to be regulated by GATA-3. However, the list of GATA-3 target genes is far from complete, and none of those that are known are expressed early enough to account for the vital role of GATA-3 at the beginning of T-cell differentiation.

Both enforced or over-expression and deletion studies rely on altering mRNA level to determine the developmental efffectors of altered GATA-3 levels, but fall short in interpreting the gene expression pattern during T-cell development in response to the control of GATA-3. This is because they cannot delineate between genes that are controlled by GATA-3 directly binding to the regulatory elements of these genes, versus genes that are influenced indirectly through a transcription factor network in which GATA-3 is involved.

In order to understand how GATA-3 directly influences gene expression patterns during T-cell development, we are investigating the potential GATA-3 target genes in two pro-T cell lines: P2C2 cells, a DN3-like cell line; and BL3a cells, an early pro-T-like lymphoma with high GATA3 expression level. We are optimizing conditions to detect GATA-3 binding sites by combining chromatin immunoprecipitation (ChIP)-based approach with genomic DNA microarrays (ChIP-chip). More specifically, GATA-3 is cross-linked to DNA at its binding sites in the context of the natural nuclear environment, and DNA-protein complex are collected via immunoprecipitation. After size-optimized DNA fragments specifically bound to GATA-3 are isolated, they will be screened using genomic microarray technology.

ChIP-chip can only provide information about a transcription factor DNA-binding status, but not about its role on the expression of the genes. Once genome-wide GATA-3 DNA-binding sites are identified, individual sites will be further studied by other biochemical and molecular methods, such as: (1) to transfect P2C2 cells with construct containing a GATA-3 binding site sequence and a reporter gene, whose expression pattern could correspond to the expression pattern of that GATA-3 target gene *in vivo*; (2) since GATA-3 is suggested to be able to induce chromatin structure, epigenetic studies on the DNA-binding sites can help reveal how GATA-3 regulates T-cell-specific gene expression; and, (3) to find other transcription factors that interact with GATA-3.

409. Use of obligate transcriptional repressors to study the role of transcription factors in T-cell development

Jerry G. Kwong, Deirdre D. Scripture Adams, Mark Zarnegar

GATA-3, PU-1, and GATA-2 are all transcription factors known to be important to T-cell development. These factors either upregulate or downregulate expression of other genes and thereby promote T-cell progression. An important question is to determine which of these effects on other important genes are direct and which are indirect. To answer this question, obligate repressor fusions of each of the above genes have been constructed in retroviral

vectors, using a repressor domain derived from the *Drosophila melanogaster* Engrailed protein fused to the DNA-binding domain of each of the above factors (see last year's Annual Report). These fusion proteins will repress gene expression wherever they are targeted by their DNA-binding domains, regardless of the original function of the normal version of the factor. We are now infecting early T cells using either these obligate repressor-encoding retroviruses, or retroviruses carrying the wild-type version of the same transcription factors, and will analyze gene expression profiles in both cases using realtime quantitative PCR. The effects on gene expression of these obligate repressors can be compared with the effects seen when the wild-type factor is expressed. A similar pattern of gene expression between wild type and obligate repressor will suggest that the normal transcription factor in general acts directly as a repressor. In this case, any gene-activating effects of the normal factor and the engrailed fusion protein must be indirect, perhaps by repressing a repressor. If the pattern of gene expression in the wild-type factor is substantially different from the fusion protein, on the other hand, this will suggest that the factor in question normally acts positively.

This strategy should greatly expand our knowledge of the genes directly regulated by GATA-3, GATA-2, and PU.1, and help us to place them correctly in the transcriptional network controlling T-cell development.

410. Gene discovery and expression analysis of T-lineage regulators: Defining the transition from hematopoietic stem cell into the T-cell pathway

C. Chace Tydell, Elizabeth Sharon David Fung, Lee Rowen[*]*, Jonathan E. Moore, Ellen V. Rothenberg*

Notch signaling activates T-lineage differentiation from hematopoietic progenitors, but only a few of the transcription factors initiating this program have been identified, e.g., GATA-3 and TCF-1. To identify additional regulators of T-cell specification, a cDNA library from pro-T cells was screened for genes specifically upregulated in pro-T cells as compared to hematopoietic progenitors. In addition to 12 known T-lineage genes, over less-characterized 75 genes of interest were identified by a stringent statistical criterion, and these were enriched for transcriptional regulators, RNA-binding proteins, and helicases. Quantitative real-time PCR confirmed that most of the ~40 genes selected for closer study are specifically upregulated in T-lineage cells relative to precursors of B or myeloid lineage. To a striking extent, however, T-lineage program components including Zn finger transcription factors, helicases, and signaling adaptors were also shared by Lin- Sca-1+ c-Kit+ (LSK) CD27- stem cells and LSK CD27+ multipotent progenitors, though not by cells differentiating in other pathways. Thus, much of the early T-lineage regulatory state is a legacy from stem cells. The few genes sharply upregulated between LSK CD27+ and pro-T cell stages included transcription factors Bcl11b and HEBalt as well as Notch target Deltex1, its relative Deltex3L, Fkbp5, Eva1, and Tmem131 (RW1). Like GATA-3 and Deltex1, Bcl11b, Fkbp5, and Eva1 were dependent on Notch/Delta signaling for induction in fetal liver precursors, while Bcl11b uniquely was upregulated between the DN1 and DN2 pro-T cell stages corresponding to T-lineage specification.

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411. Non-homogeneity in T-cell developmental progression: Using single-cell culture to look at stochastic and non-stochastic models of developmental stage transition and fate choice

Deirdre Scripture Adams

In the thymus, as well as in the most powerful and well used *in vitro* model of T-cell development, the OP9-DL1 culture system, T-cell precursors do not progress through development in a uniform mass, even when sorted to apparent homogeneity: They instead pass through each successive developmental state spread among several consecutive stages, or alternatively, among "neighboring" cell fates. We have found that this is the case even at the level of progeny of a single cell. A single cell can give rise to daughters which have differing rates of progression along a given developmental pathway, or which have made divergent fate choices. Fetal liver precursors have been used successfully to begin to address these questions when sorted as individual precursors directly on to OP9-DL1 stromal layers and cultured while monitoring expansion, divergence, and progression along the T pathway. We are using the OP9-DL1 *in vitro* T-cell development system to generate large data sets to explore variances in these behavior types that differ quantitatively among different developmental states.

412. *cis*-Regulatory analysis of the hematopoietic transcription factor PU.1

Mark Zarnegar

Developmental regulation of transcription factor expression is critical in many cell fate decisions. In developing T cells, many factors are up regulated, while some are turned off, as the cells progress from early thymic precursors (DN1 and DN2) to cells committed to the T-cell fate (DN3). Our lab has demonstrated that forced expression of PU.1 in thymic precursors, where its expression is normally turned off, arrests the cells and prevents them from becoming committed to the T-cell lineage. We therefore seek to understand how PU.1 expression is controlled in developing pro-T cells through mapping of the relevant *cis*-regulatory elements.

Comparing the sequences of the mouse and human loci, we have identified several pockets of **C**onserved **E**lements (CE1-9) upstream of exon 1 that may function to regulate PU.1 transcription. DNase hypersensitivity (DHS) and chromatin immunoprecipitation assays (ChIP), both techniques that can identify regions of transcription factor accessibility, and thus potential regulatory function, have been used to examine the conserved elements. Using our mapping

information from the ChIP and DNase HS data, we postulated that region CE8-9 may contain a non-specific regulatory element, while CE3-7 may contain a myeloid-specific enhancer element, and/or a T-cell specific regulatory region (see last year's Annual Report).

We have designed luciferase reporter constructs to test for cell type-specific regulatory function of these upstream regions. CE9-8 acts as a non-specific enhancer in multiple cell lines. CE5 functions as an enhancer only in myeloid cells. CE4 contains a T-cell specific silencer region. We also have observed a non-conserved region between CE4 and CE3, CE3A, which can also act as T cell silencer. Complete mapping of this second repressive element is currently underway. We are also making stable cell lines in order to confirm the elements function similarly when integrated into chromatin.

Much effort has gone into identifying the transcription factors that bind and thus regulate the activity of the potential regulatory elements. Electrophoretic mobility shift assays implicate two important transcription factor families, Ets and Runx, in the potential regulation of PU.1. We have shown different Ets family members can bind to CE8 in PU.1 expressing versus non-expressing cell lines, perhaps contributing to the different PU.1 expression patterns in these lineages. Gel shifts have also confirmed the ability of Runx family members to bind a site present in CE4. When we knock down Runx1 by cotransfecting antisense morpholinos in our transient transfection system, we inhibit the silencing effect of CE4. We are attempting to identify other factors that may act in combination with Runx1 to control CE4's silencing ability. We have yet to identify a mechanism through which the alternative silencer, CE3A, may be acting. Several potential transcription factor-binding sites are present in CE3A, including sites for Gfi1, Ikaros, Bcl11B, and ZEB (Zfhx1a), all factors known to be able to repress transcription of target genes. Gel shifts to examine binding to these sites are underway.

413. Finding regulatory elements of PU.1 in an embryonic stem cell differentiation system

Long Li, Mark Zarnegar

PU.1, an ETS family transcriptional factor, plays an important role in hematopoiesis: Aberrant expression of PU.1 may result in disruption of some blood lineages or even cause leukemia. Highly regulated expression of PU.1 is critical for T-lymphocyte differentiation: Although essential for the earliest stages, its expression decreases dramatically during T-cell specification and is not detectable after T-cell differentiation reaches DN3; continued expression of PU.1 blocks further differentiation and even redirects cells into myeloid fate. Decreasing of PU.1 expression coincides with loss of developmental potential of pro-T cells to give rise to B, NK or myeloid cells. As described in the previous abstract, we are seeking to map the regulatory elements responsible for cell-type-specific differences in PU.1 expression, but the standard cell-line models cannot reproduce the dynamic changes of PU.1 expression in T-cell differentiation and their strict temporal order that we seek to explain. Therefore, we are developing an embryonic stem cell differentiation system to score activity of regulatory elements that control developmental changes in PU.1 expression.

Co-culture of fetal liver hematopoetic stem cells (HSC) with OP9-DL1 cells is being used in our group to generate T cells from these HSC. We are going one step further by using the co-culture of embryonic stem (ES) R1 cells with OP9/OP9-DL1 to generate B cells, myeloid cells and T cells directly from embryonic stem cells *in vitro*. The advantages of this new system include: (1) It enables activity of a regulatory sequence reporter construct to be measured from the same integration event in divergent cell lineages, without going through a transgenic mouse; and (2) It enables us to monitor the change in activity of a specific regulatory element at different time points during T-cell development. These features should make the embryonic stem-cell differentiation system a powerful approach to assay the function of crucial regulatory elements (conserved DNA sequences, hotspots for DNA methylation and histone modifications) that control the different expression patterns of PU.1 across different hematopoetic lineages. Currently we are optimizing the conditions for this embryonic stem-cell differentiation system. In parallel, to look for direct molecular signatures of repression events, we are mapping differences in DNA methylation and histone H3-Lys 27 tri-methylation around an upstream repressor sequence CE3A in DN3-like P2C2 cells, which do not express PU.1, and in 32Dcl5, an IL-3-dependent pre-mast cell line which does express PU.1. Future experiments include: (1) To transfect ESR1 cells with constructs containing conserved DNA sequence in upstream of PU.1 to find enhancer or repressor sequences; (2) To find changes of DNA methylation and histone modifications of PU.1 during the DN1 and DN2 stages when its expression down regulates; and, (3) To find how conserved DNA sequences, DNA methylation and histone modifications cooperate to fine-tune the expression of PU.1.

414. Characterization of early T-cell development in autoimmune non-obese diabetic (NOD) mice

Mary Yui, Ni Feng

Type 1-diabetes (T1D) in non-obese diabetic (NOD) mice and humans is a complex polygenic autoimmune disease, which results from T cell-mediated destruction of insulin-producing pancreatic beta cells. It is the balance between different functional T-cell populations that determines autoimmunity, as T cells are not only crucial to disease pathogenesis, but also to the control of ongoing autoimmunity. Several functional defects have been reported in the immature and mature αβ-T cells, NKT and NK cells of NOD mice, which may contribute to disease susceptibility. Because all T-cell subsets and NK cells share common precursors, we hypothesized that some key defects affecting fundamental T-cell functions will manifest themselves in the early T-cell progenitors. Our previous studies have shown that NOD mice, with

mutations that prevent TCR rearrangements, spontaneously violate the earliest checkpoint in T cell development, β-selection. This defect in early T cell differentiation programming in NOD mice occurs at the time that T cells first use the T-cell receptor (TCR) and its signaling pathways. Furthermore, the trait genetically maps to know diabetes susceptibility loci, suggesting a role in disease.

Using the OP9 Delta-like 1 (OP9-DL1) co-culture system, which permits *in vitro* T-cell development, we have found poor αβ-T-cell development from NOD T-cell precursors, while γ -T-cell development appears to be enhanced. Because the strength of TCR signaling can affect this lineage choice, this result is consistent with the results from NOD.*Rag*$^{-/-}$ mice, which cannot rearrange a TCR, suggesting abnormally high levels of spontaneous pre-TCR signaling at the β-selection checkpoint. We have more recently found that surface receptors that can modulate the strength and nature of TCR signals (e.g., CD4, CD2 and CD5), as well as co-stimulatory receptors involved in T-cell survival and proliferation (e.g., CD27 and CD28), are expressed at significantly higher or lower levels in NOD and NOD.*Rag*$^{-/-}$ early T cells as compared with their B6 and B6.Rag$^{-/-}$ T-cell counterparts, which may play a role in our *in vitro* observations. We plan to use the OP9-DL1 system for more detailed kinetic analyses to determine differentiation, survival and proliferation rates, as well as sensitivity to cytokine growth factors and Notch signals, for the development of various T-cell subsets.

415. Genetic dissection of the early T-cell checkpoint breakthrough in NOD mice using congenic mice

Mary Yui, Ni Feng

Based upon genetic analysis of Type 1 diabetes in NOD mice, over 20 diabetes susceptibility loci have been mapped. However, very few of the genes involved in disease have yet been identified. Based upon our previous observations that immunodeficient NOD-*scid* and –*Rag*$^{-/-}$ mice, which cannot rearrange T-cell receptors, spontaneously break through the first T-cell developmental checkpoint, we wished to determine whether or not this fundamental defect in NOD T cells maps to know diabetes susceptibility genetic regions. This checkpoint violation provides a fairly simple phenotype amenable to genetic analysis, in a mouse that has all of the diabetes susceptibility genes, but does not have the complications of ongoing autoimmune disease. As previously reported, a linkage analysis was conducted using PCR-based microsatellite repeat polymorphisms to map the gene(s) responsible for the trait. (NOD X B6) X NOD-*scid/scid* backcross mice were phenotyped and then polymorphisms genotyped in the genomic regions including 17 diabetogenic loci found on seven chromosomes. We found that the trait appears to map closely to regions within genetic intervals on chromosomes 4 and 2 containing major diabetes susceptibility loci. The chromosome 4 genetic region is of particular interest as the 2-3 diabetogenic genes within it are thought to specifically affect T-cell functions. Although these susceptibility genes remain unidentified, congenic mice developed by Linda Wicker and colleagues (Cambridge, UK), in which the genetic interval is replaced by a corresponding resistant genetic interval from C57Bl/10 mice, resulted in a reduction in the incidence of diabetes from 80% to 3%. Furthermore, T cells were found to infiltrate but not destroy the pancreas in these congenic mice, suggesting that the genes within this region may control the balance between pathogenic and regulatory self-reactive T cells. We have obtained these congenic mice and are currently carrying out crosses to make them *Rag*-deficient to determine whether or not genes within this diabetes susceptibility region are critical for the β-selection checkpoint violation. Furthermore, we are characterizing early T-cell development in these congenic mice to determine whether or not any of the differences observed between diabetes-prone NOD and resistant C57Bl/6 mice also map to this genetic region.

416. Analysis of the expression of key T-cell genes involved in early T-cell developmental programming in NOD mice

Chen Yee Liaw, Mary Yui, Ni Feng

Detailed analysis of the β-selection checkpoint violation in NOD.*Rag*$^{-/-}$ mice shows a derangement in the timing and level of expression of several developmentally regulated cell surface receptors. To elucidate the molecular mechanisms of the aberrant development and checkpoint violation in NOD.*Rag*$^{-/-}$ T cells, early T-cell populations have been purified from the thymuses of diabetes-prone NOD and normal C57Bl/6 mice, both *Rag*-deficient and wild type, using antibodies to specific cell surface markers and sorting by flow cytometry. RNA has been extracted from these cells and reverse transcribed into cDNA for gene expression analysis using real time quantitative PCR. Based upon previous work in our lab, we have selected a set of transcription factor and effector genes to be analyzed that are key to T-cell developmental programming and are normally modulated during this time. We hope to identify specific genes that are aberrantly expressed in the in early T cells from NOD mice as a clue to the defective pathways and genes involved in the alterations in T development, including the β-selection checkpoint violation.

417. Conditioning the development of antigen-presenting cells by exposure to neutrophil granule proteins

C. Chace Tydell

The "Hygiene Hypothesis" addresses the evidence that the mammalian immune system requires exposure to bacterial or parasitic products for normal development (1-3). The consequence of first-world hygiene has been linked to the high incidence of asthma, Type-I diabetes, Hodgkin lymphoma and IBD (inflammatory bowel disease) in developed nations (4-6). In a controlled environment, the incidence of Type-I diabetes in colonies of NOD mice is markedly impacted by hygiene conditions.

Only recently has the role of the innate immune system been explored for its significance in immune system homeostasis. Specifically, bacterial exposure commonly induces a successful response by the cellular arm of the innate immune system; the mustering of neutrophils to a site of challenge with subsequent discharge of large amounts of biologically active neutrophil granule proteins. The proteins of neutrophil granules are not only antimicrobial in activity but have diverse effects on host cells (7-10). It is our hypothesis that the mechanism for the hygiene effect lies at the interface of the innate and adaptive immune systems. Therefore, we have begun to investigate the effect of neutrophil granule proteins on the programming of two types of antigen presenting cells, dendritic cells and macrophages, which also present vital instructive cues to cells of the adaptive system. Two neutrophil granule proteins, lactoferrin and PGRP-S, are of particular interest.

The first specific neutrophil granule protein we considered for a possible effect on dendritic cell maturation was lactoferrin. A prominent component of neutrophil secondary granules, lactoferrin, is not potently microbicidal; however, numerous studies attribute immunomodulatory properties to lactoferrin (11). Analysis of sorted populations of macrophages and dendritic cells by qRT-PCR demonstrated that both cell types express high levels of Lrp1, a putative lactoferrin receptor (11-13). Therefore, the effect of lactoferrin on dendritic cells, obtained from mouse bone marrow using the standard culture protocol, was tested. Development of dendritic cells, cultured in the presence of GM-CSF with or without lactoferrin, were analyzed by FACs with dendritic cell markers, and their maturation in the presence of LPS was evaluated. Cells developed in the presence of the anti-inflammatory cytokine IL-10 were also analyzed for comparison. Cells cultured with IL-10 show a relative resistance to LPS-induced maturation. DCs generated in the presence or absence of lactoferrin, are substantially similar when analyzed by flow cytometry with antibodies against a variety of maturation markers. When sorted populations of DCs are further evaluated for gene expression patterns by qRT-PCR, these patterns are, again, mostly indistinguishable. To date we have tested the expression of Hhex, CD86, IL-10, Lmo4, Id2, CAM1, PGRP and lactoferrin in CD86+ and CD86- LPS-exposed dendritic cells. However, unexpectedly, IL-12 expression was shown to be affected by the presence of lactoferrin in the culture medium. This is important because IL-12 secretion by DCs in an immune response has a powerful effect on the differentiation of naïve T cells to different effector types. Expression of IL-12 was high in CD86- LPS-exposed DCs in all three media. Expression levels downregulated more than 100 fold upon DC maturation (as indicated by CD86 surface expression) in the control and IL10 cytokine media but did not downregulate in the lactoferrin cultured DCs. Initial studies thus indicate that this abundant neutrophil granule protein is affecting dendritic cell development, possibly generating "semi-mature" dendritic cells, reported to induce antigen-specific protection from autoimmunity (14). Experiments to confirm and extend these observations are in progress.

The limitation of the established technique of culturing murine dendritic cells from bone marrow, using GM-CSF, is that it generates a heterogeneous population of myeloid cells. Along with dendritic cells, these cultures generate high percentages of macrophages and neutrophils, precluding any media condition from being "lactoferrin free." Only recently, with the identification of a specific precursor cell for dendritic cells and macrophages, termed MDPs (15), has an *in vitro* exploration of the effects of neutrophil granule proteins on macrophage and dendritic cell development been possible. These MDPs are CX3CR1+ ckit+ BM progenitors that cannot develop into neutrophils. We have developed a technique to sort MDPs to generate neutrophil-free cultures of dendritic cells (with GM-CSF) or macrophages (with M-CSF). The effect of neutrophil granule proteins on the development of precursors guided toward a dendritic cell or macrophage fate may now be analyzed. We propose to analyze lactoferrin and PGRP-S in this novel culture system. In addition, the impact of IL-10 on these heterogeneous BM cultures has been documented, so it would be valuable to analyze the specific effect of IL-10 on pure cultures of dendritic cells.

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418. Regulatory analysis of the antigen receptor genes of lamprey lymphocytes

Jonathan E. Moore

Our lab also researches the evolution of the lymphocyte lineage, its genetic regulation, and its development. Previous research in our laboratory has shown that the regulation of the adaptive immune system within the gnathostomes, or jawed vertebrates, is remarkably conserved. For example, between mammals and skates, the most basal of the gnathostome phyla, nearly every transcription factor involved in T- and B- lineage development is conserved, as is also its approximate expression pattern. We are in the midst of pushing our understanding of the evolution of this system one critical step further to the agnathans, or jawless vertebrates.

Though the agnathans have many of the cellular-and organismal-level hallmarks of the adaptive immune system, there is no evidence they have its genetic hallmarks such as genes for antibodies, recombinant T-cell receptors, MHCs, or Rag-mediated recombination. The beginning of the resolution to this seeming conundrum was announced in 2004 by our collaborators, Drs. Zeev Pancer, Chris Amemiya, and Max Cooper, who discovered an alternative highly diverse recombinant protein, the variable lymphocyte receptor (VLR), expressed on the surface of agnathan lymphocytes. It is tantalizing to hypothesize that the transcription regulatory network involved in immune regulation originated before the last common ancestor of the vertebrates. In order to investigate this possibility, we are endeavoring to understand the transcriptional regulation of the VLR.

This past year, we refined our bioinformatic assays of the upstream region of the lamprey VLR gene, we have created a myriad of luciferase reporter constructs incorporating segments of this region, and assayed their activity in several heterologous cell lines with and without some cotransfected expression constructs of immunological transcription factors. Despite the evolutionary distances involved, expression levels in these reporter constructs are quite high compared to background. One tantalizing preliminary result shows considerable activation in a mammalian B-cell line by a region approximately 1 to 2 kb upstream of the transcription start site; interestingly, this region is rich in predicted GATA and Pax-family-binding sites.

Additionally, we have isolated by flow cytometry VLR-positive cells from lampreys; in the near future, we will make cDNA from these and prepare nuclear extracts from similarly obtained samples. We have also taken preliminary steps to resolve the controversial phylogenetic relationship between the hagfish, lamprey, and gnathostomes, a question with some considerable relevance to this research. Last, we have created a statistical comparative sequence analysis tool that we hope to generalize and make available to others.

Rothenberg laboratory publications

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Summary: Our work in the Alliance for Cellular Signaling has led us to a view of the architecture of the signaling circuits that process information gleaned at the surface of the cell. After examining the responses to 22 different ligands applied to the cell singly or in pairs, we concluded that the network that coordinates information is relatively *simple*. Most circuits lead fairly directly from a cell surface receptor through a signal transduction process to a response regulator which effects cell metabolism. However, approximately 10% of the circuits involve more complex and unpredictable responses, "non-additive responses." The results of the initial Alliance experiment points to these circuits that include "interaction agents." We have proposed to find the molecular nature of these interacting elements in the subset of the signaling network that processes information mediated by the G protein system. It is necessary to first generate the tools that will allow us to precisely define the components of this circuitry. Our work over the past two or three years has culminated in just such a "tool box." We built a series of vectors that can conditionally express single or multiple miRNA scaffoldings that include RNAi sequences targeted at silencing the production of specific gene products. These are versatile single lentiviral vectors that can be used both to transform or transfect tissue culture cells and also to transfect primary cells. The vectors have features that allow us to block specific gene expression and to restore that expression. Vectors can also be equipped with mutant forms of a gene so that the native form can be silenced while the alternative form is expressed. The second set of tools developed over the last year and that now are beginning to be deployed involve the use of microfluidics. Robert Bao (from Dr. Steve Quake's laboratory) has built a "lab on a chip" that allows us to design complex experimental protocols for the study of cells responding to different ligands applied at different times, singly or in pairs, under conditions that are identical for each of the 6-12 small populations of approximately 100 cells each and to derive data for both single cell responses and for the response of each small population. We are using this approach to study the mechanisms involved in a number of responses including receptor desensitization and ligand interactions that involve the ultimate release of calcium in the cell. We have together with colleagues at University of California, San Francisco, begun to develop methods for transfecting bone marrow derived macrophages (BMDM) so that we can obtain primary cells that also carry the gene silencing vectors and can be tested for their activity.

In addition, we have continued our work in collaboration with Drs. David Anderson and Allan Basbaum trying to understand the function of G protein pathways in the small diameter nociceptor neurons that innervate the skin in the mouse. Dr. Sang-Kyou Han in our lab has found that mutants that are devoid of PLC β3 also lose their scratching intradermal response to injections of histamine. He has been able to show that that the "itch" response to injected histamine and a variety of agonists and antagonists that either bind to the histamine H1 receptor or induce mast cell degranulation, lose their ability to induce itching in PLC β3 defective mice. He has been able to show that the histamine H1 receptor that mediates the response is localized on a fraction of the small diameter nociceptor neurons that ordinarily specifically express PLC β3. This work is being pursued to further understand the basis for the function of subsets of these nociceptive neurons.

Finally, in collaboration with Dr. Mircea Podar, we have undertaken a computational study of the two component signaling systems in microorganisms and particularly the nature of the two component systems that have been discovered through metagenomic sequencing. We plan to use computational tools to understand the phylogenetic distribution of different two component signaling systems, their functional distribution, and their correlation with environmental responses.

419. Molecular Biology Laboratory of the Alliance for Cellular Signaling (AfCS)

Iain Fraser, Joelle Zavzavadjian, Leah Santat, Jamie Liu, Estelle Wall, Adrienne Driver, Kum Joo Shin, Mi Sook Chang, Jong Ik Hwang, Baljinder Randhawa, Melvin Simon

Studies by the Alliance for Cellular Signaling (AfCS) in the RAW264.7 mouse macrophage cell line are focused on the analysis of crosstalk between signaling pathways when the cell is challenged with multiple ligand stimuli. The AfCS ligand screens have identified thousands of interactions between signaling pathways that provide insight to the mechanisms used by the cell to respond appropriately to extracellular stimuli (1). RNAi provides a powerful method to investigate the molecular basis of interaction between signaling pathways, and we

have developed sophisticated methods for the application of RNAi in RAW cells and primary macrophages. We have developed a lentiviral vector platform (pSLIK; Single Lentivector for Inducible Knockdown) that permits Tet-regulated expression of 'microRNA-like' shRNAs (miR-shRNA) from a single viral infection of any naïve cell system (2). In mouse embryonic fibroblasts, the pSLIK platform was used to conditionally deplete the expression of the heterotrimeric G proteins, Gα12 and Gα13, both singly and in combination, demonstrating the Gα13 dependence of SRE-mediated transcription. In RAW264.7 macrophages, regulated knockdown of Gβ2 correlated with a reduced Ca2+ response to C5a. Insertion of a GFP transgene upstream of the Gβ2 miR-shRNA allowed concomitant re-expression of a heterologous mRNA during Tet-dependent target gene knockdown, significantly enhancing the experimental applicability of the pSLIK system. We are currently using these and other experimental tools, in collaboration with the other AfCS laboratories at UCSF, UTSW and Berkeley, to develop mechanistic models of signal transduction pathways in the RAW264.7 cell line.

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420. Phospholipase Cβ 3 mediates the scratching response activated by the histamine H1 receptor on C-fiber nociceptive neurons

Sang Kyou Han, Valeria Mancino, Melvin I. Simon

A variety of extracellular signals are transduced by G-protein coupled receptor (GPCR) activated circuits that include the enzyme phosphoinositide-specific phospholipase Cβ (PLCβ). There are four isozymes of PLC-β, β1-β4, and some of these isoforms mediate sensory modalities such as taste, vision, and pain sensation in a variety of organisms. Here, we investigated the nature of the function of PLCβ isozymes in primary sensory neurons by using mutant mice deficient for specific PLCβ isoforms. Expression analysis indicated that PLCβ3 and β4 were expressed in different subsets of sensory neurons. PLCβ3 is predominantly expressed in a sub-population of C-fiber nociceptors, and anterogradely transported to peripheral nerve terminals. Ca^{2+} imaging studies revealed that PLCβ3, but not PLCβ4 specifically mediated histamine induced calcium responses in cultured DRG (Dorsal Root Ganglia) neurons and the responses were completely abolished by the histamine H1 receptor antagonist, mepyramine. In line with this, we found that PLCβ3-/- mice showed significant defects in scratching behavior induced by histamine, HTMT (histamine-trifluoromethyl-toluidine), a selective H1 agonist, as well as by compound 48/80, a mast cell activator and to a lesser extent by clobenpropit, an H4 receptor agonist and H3 receptor antagonist. These results demonstrate that PLCβ3 is required to mediate "itch" sensation in response to histamine acting on the histamine H1 receptor in C-fiber nociceptive neurons.

421. A microfluidic platform for cell signaling assays

Robert Bao

We are working to establish an experimental platform for probing calcium responses in mammalian cells within microfluidic devices. Using pressure-actuated microvalves, we can precisely gate and switch flow to cells anchored to microchannel floors. Because the devices are permeable to oxygen and carbon dioxide, cells can be cultured in the device for up to several days under conditions that effectively mimic those found in a cell culture incubator. Calcium responses in hundreds of individual cells can then be imaged through the device using standard epifluorescence microscopy. The use of microfluidic devices has several advantages: (1) we have excellent control over perfusion conditions that the cells experience, both during culture and experiment; (2) many separate experiments may be incorporated into a single microscope field-of-view, so that several different conditions, including positive and negative controls, may be integrated into a single experimental data acquisition run; and, (3) cells can be kept in culture between different experiments, so that long-term, cell-to-cell variabilities in responses to different ligands can be probed; and, (4) reagent volume requirements for cell stimulation are much lower than those in conventional cellular assays, so there are significant potential cost savings for expensive reagents.

We are currently working with a device design with six separate channels for use during experiment. Preliminary results for RAW macrophages stimulated with UDP are comparable to those seen in single-cell experiments performed in macroscopic wells. Work is still ongoing to probe and understand the effects of flow rate and shear stress, both during cell preparation and ligand stimulus, on cellular calcium responses. In addition to the general scientific interest, understanding these issues promises to improve data quality and consistency for many experiments involving solution exchange.

Publications

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Summary: We seek to understand how the genome controls the development, behavior and physiology of *C. elegans*. Our main foci are on signaling pathways and transcriptional regulation. Our approaches are experimental, computational and synthetic. Specifically, we use molecular genetics to understand detailed mechanisms, and functional genomics to obtain global views of development and behavior. We take computational approaches to understand signal transduction, developmental pattern formation and behavioral circuits. We try to couple tightly computation and experimental data, in part to use computation to make experimental tests more efficient. Moreover, we study other genomes, genetics, and biology of other nematodes to help us comprehend *C. elegans*, to learn how development and behavior evolve, and to learn how to control parasitic and pestilent nematodes.

In the area of signal transduction, we continue to define pathway interactions and to understand the determinants of signaling specificity: How does the same pathway lead to distinct outcomes in different tissues? For these studies we analyze EGF-receptor signaling, WNT signaling, TRP channels, and G protein-mediated signaling pathways. We have found a new role for EGF signaling, controlling behavioral quiescence during development.

Vulval development involves a remarkable series of intercellular signaling events that coordinate the patterning of the uterine and vulval epithelia and allow them to connect precisely. Specification of the anchor cell from the ventral uterine epithelium breaks the symmetry of the gonad. The anchor cell then produces the vulval-inducing signal, LIN-3, an epidermal growth factor like protein that acts via *C. elegans* homologs of EGF-receptor, RAS and MAP kinase. Inductive signaling is regulated at the level of ligand production, as well as the responsiveness of the receiving cells. LIN-3 is produced in a highly localized and regulator manner. After the anchor cell induces the vulva, a complex program of further pattern formation, cell type specification and morphogenesis follows. The primary (1°) vulval lineage generates an E-F-F-E pattern of cell types while the 2° vulval lineage generates an A-B-C-D pattern of cell types. We now have our hands on a number of receptor proteins, transcription factors and regulated genes; we are trying to define this regulatory network to understand how organogenesis is genetically programmed. The anchor cell recognizes one of the seven vulval cell types and invades the vulval epithelium in a process akin to tumor metastasis, and we have found genes necessary for this process. Regulation by the EGF-receptor, WNT and HOM-C pathways impinge not only on vulval development but also the neuroectoblast P12 specification, male hook and spicule development. By comparing these examples with vulval development, we seek to understand the signaling specificity and signal integration. The relative contributions of EGF and WNT differ in each example. Multipotent cells with three fates utilize NOTCH signaling, as well as EGF/WNT signaling

Our efforts in genomics are experimental and computational. Our experimental genome annotation includes identifying *in vitro* binding sites for transcription factors, testing enhancer-function in transgenic worms, and systematic inactivation of *C. elegans* transcription factors. We are investigating ways to compare the genomes of *Caenorhabditis* species. In particular, we are collaborating with the Genome Sequencing Center of Washington University to annotate new nematode genomes. Our computational projects involve establishing pipelines for *cis*-regulatory computational analysis, new programs to use orthology and known binding sites or motifs, etc. We have successfully combined information from worms, flies and yeast to predict gene-gene interactions in *C. elegans*, and are extending this to other species.

We are part of the WormBase consortium, which develops and maintains WormBase, a web-accessible comprehensive database of the genome, genetics and biology of *C. elegans* and close relatives (www.wormbase.org). We initiated WormBook, an open-access online text of *C. elegans* biology associated with WormBase. We have developed Textpresso (www.textpresso.org), an ontology-based search engine for full text of biological papers. Textpresso is used by *C. elegans* researchers, as well as the WormBase staff. It is being expanded to other organisms and fields of study, in particular Neuroscience and *Drosophila*. We are also part of the Gene Ontology Consortium

(www.geneontology.org), which seeks to annotate gene and protein function with a standardized, organized vocabulary. WormBase, Gene Ontology and Textpresso are part of the Generic Model Organism (GMOD) Project (www.gmod.org), a collaborative project among organism-specific databases to develop generic software.

Our behavioral studies focus on understanding male mating behavior, as well as locomotion of both sexes. For specific projects we study egg laying, feeding, chemotaxis, osmotic avoidance among other simple behaviors. Mating behavior, with its multiple steps, is arguably the most complex of *C. elegans* behaviors. Because it is not essential for reproduction, given the presence of internally self-fertilizing hermaphrodites, male mating is useful to elucidate how genes control behavior. We are studying several aspects of male mating behavior to understand the neuronal circuits that control the behavior and how they are genetically encoded. Our comparative studies include both analyzing behavioral differences among species, and genetic analysis of *C. briggsae*, *Pristionchus pacificus* (a nematode species we discovered during the 1990s), and *Heterorhabditis bacteriophora* (an insect-killing nematode).

We have developed a machine-vision system that automatically quantifies the locomotion of nematodes. We use this system to study individual genes, to examine epistatic interactions among genes, and to obtain data to support mathematical modeling efforts. We are expanding this effort to analyze mating behavior of nematodes. We are involved in efforts to model worm movement, vulval pattern formation and aspects of signal transduction.

422. *cis*-Regulatory analysis of three *Caenorhabditis* genomes

Erich M. Schwarz, Ali Mortazavi, Steven G. Kuntz, Alok J. Saldanha, John A. DeModena, Daniel Fu, Tristan De Buysscher, Hiroaki Shizuya, Joseph C. Roden[1], Barbara J. Wold[2], Paul W. Sternberg

We scanned three *Caenorhabditis* genomes (*C. elegans*, *C. briggsae*, and the newly sequenced *C. remanei*) for conserved non-coding DNA elements that could indicate *cis*-regulatory signals. We first used MUSSA, an n-species generalization of FamilyRelations, to select ungapped blocks of high identity. MUSSA identified ~2% of the non-coding DNA in the *ceh 13/lin 39* Hox cluster as conserved between *C. elegans*, *C. briggsae*, and *C. sp. CB5161*. Within this restricted subset, transgenic assays of Hox cluster elements show expression patterns for five of seven tested. Fine-scale examination of other experimentally verified *cis*-regulatory elements in *lin 3* and *lin 11* shows that they are closely associated with MUSSA-identified elements, but not identical to them. We hypothesized that this loose association of small regulatory motifs with large conserved blocks will be generally valid. We, thus, carried out a genome-wide analysis with the Cistematic open-source framework. Cistematic used MUSSA to find blocks of non-coding DNA in the flanks and introns of 8,100 genes with orthologs in all three genomes. These blocks were then scanned for up to five-candidate position-weight matrices (PWMs) per gene with MEME, and the PWMs were purged of degenerate and redundant instances. The resulting 16,385 PWMs were used to re-scan all three genomes, checking for conservation and significantly associated GO terms; this biased the motif finders towards conserved motifs in MUSSA blocks, but allowed genome-wide analyses to be unrestricted by MUSSA. We found 1045 conserved motifs associated with defined biological functions (as indicated by Gene Ontology terms, anatomical expression patterns, or homology groups), which we are now analyzing for coexpression by microarray and anatomical data in WormBase. We also tested *in vivo* Cistematic's ability to find regulatory elements in coexpressed collagen genes; deletion of Cistematic-predicted elements from collagen promoter regions reliably altered their regulation of reporter genes.

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423. Genome-wide prediction of *C. elegans* genetic interactions

Weiwei Zhong

An essential part of understanding how a genome specifies the properties of an organism is elucidating interactions among its genes. One successful method to identify genetic interactions is by modifier screens (e.g., synthetic lethal screen). However, this process is limited to easily detectable phenotypes. Metazoan biological processes often involve phenotypes that are too complex to score in large-scale screens. To study functional interactions in these processes, an alternative method, direct testing of candidate genes, is often used. Unfortunately, a genome of 20,000 genes has as many as 200 million pairwise combinations, composing a formidable challenge for experimental testing. To obtain a global view of the genetic interaction network, we took a computational approach to statistically integrate genetic data (such as expression and phenotype) from multiple species to predict genome-wide genetic interactions. We applied the approach in the nematode *Caenorhabditis elegans* and predicted 18,183 interactions among 2,254 genes. We experimentally tested a subset of our predictions and successfully identified 12 new modifiers of the *C. elegans ras* gene *let 60* and two new suppressors of the inositol-1,4,5-trisphosphate receptor (IP_3R) gene *itr 1*. Our predicted genetic interaction network provides a genome-level understanding of gene functions and a candidate "guidebook" for experimental testing.

242. EGF signaling regulated behavioral quiescence in *C. elegans*

Cheryl Van Buskirk

EGF signaling plays many roles in development across species and recently has been shown to regulate circadian activities in mammals. We have found that EGF overexpression has strikingly similar effects on behavior in *C. elegans* and mice. We have observed that overexpression of the EGFR ligand LIN-3 from an inducible promoter at any stage causes a complete and reversible cessation of feeding and movement. We have found these behavioral defects to be mediated by the EGF receptor (LET-23), by PLC-γ, and by targets of diacylglycerol (DAG) signaling, including those that mediate neurotransmitter release, suggesting that EGF signaling acts to control neurotransmission. We are currently investigating the neural circuits involved in this effect, and screening the genome for additional genes that play a role, with the aim of understanding more about how EGF affects behavior in both *C. elegans* and mammals.

425. Spatial and temporal coordination of organogenesis

*Ted Ririe, Si Hyun Kim**

Organ development requires a network of factors that direct both spatial and temporal patterning. The *C. elegans* vulva is an attractive model for studying organogenesis because of its relative complexity, and the availability of several cell fate markers whose spatial and temporal expression pattern is known. A skeletal network of the transcription factors that govern differentiation of the cells that make up the vulval have been defined. All the genes that are known to regulate differentiation of the vulval cells are functional in multiple cell types. Consequently, the mode of cell-type specific regulation remains elusive. To elucidate the mode of cell-type specific regulation we have screened for transcription factors, using RNAi, that differentially regulate *zmp 1::gfp* expression in vulA versus vulD and E cells. Also, the minimal element sufficient for *zmp 1::gfp* expression in the vulva has been defined. The alignment of *C. elegans*, *C. briggsae* and recently *C. ramanei* sequences, has revealed conserved sequences and motifs between the three species. Site-directed mutagenesis of these motifs will help elucidate the *cis*-regulation of *zmp 1* expression in individual vulval cells.

**Caltech undergraduate*

426. Tailless ortholog *nhr-67* regulates patterning and differentiation of *C. elegans* vulval cell types

Jolene S. Fernandes

Progression through *C. elegans* vulval development leads to the generation of seven distinct vulval cell types (vulA, vulB1, vulB2, vulC, vulD, vulE and vulF), each with its own unique gene expression profile. The mechanisms that establish the precise spatial patterning of these mature cell types are largely unknown. Dissection of the gene regulatory networks involved in vulval patterning and differentiation would help us understand how cells generate a spatially-defined pattern of cell fates during organogenesis. To identify components of the regulatory network underlying vulval development, we conducted RNAi screens of about half the known or predicted transcription factors and assayed for the effect on expression of vulval cell fate markers. From this screen, we identified the tailless ortholog *nhr 67* as a regulator of gene expression in multiple vulval cell types. *nhr 67* exhibits a dynamic expression pattern in the vulval cells, and interacts with three other transcriptional regulators -*cog 1* (Nkx6.1/6.2), *lin 11* (LIM) and *egl 38* (Pax) - to generate the composite expression patterns of their downstream targets. We demonstrate that the interactions between these regulatory genes are complex and vary among the seven cell types. We also discovered a striking regulatory circuit that exists in a subset of the vulval lineages: *cog* 1 and *nhr 67* cross-inhibit both one another and themselves in specific vulval cells. We postulate that the differential levels and combinatorial patterns of lin-11, cog-1 and *nhr 67* expression are a part of the regulatory code for the mature vulval cell types.

427. Cell fate, cell polarity, Wnt and Ror

Jennifer Green

Proper formation of the *C. elegans* hermaphrodite vulva results from the invariant pattern of induction and specification of three vulval precursor cells (VPCs) and depends on the integration of the Ras, Wnt, and Notch pathways. We are investigating the role of *cam 1*, the *C. elegans* homolog of the Ror family of receptor tyrosine kinases (RTKs), in VPC induction. Ror proteins contain extracellular Frizzled, Kringle, and Immunoglobulin domains and an intracellular tyrosine kinase domain. The ligand and downstream signaling components of Ror proteins are unknown at this time. We have identified *cam 1* as a negative regulator of the vulval induction process and are attempting to elucidate its relationship with the above pathways. In addition to regulating cell fate, Wnt signaling also orients the polarity VPC divisions. We are investigating how different Wnts interact to establish default polarity, basal polarity, and reversed polarity to generate a mirror-symmetric organ.

428. EGF signaling specificity

Adeline Seah

LET-23(EGFR) activates two signal transduction pathways: RAS-MAPK for P11/P12 cell fate determination, vulval development, larval viability and male spicule development and IP_3-Ca^{2+} for ovulation. It is not known what determines the specificity among the Ras-MAPK-dependent inductions. One possibility is that different transcription factors act in parallel to or downstream of the EGF signal in different tissues. Hox genes have been implicated as one class of regulatory elements that confer specificity to the EGF pathway in P11/P12 cell fate determination and vulva development. However, their role in larval viability and spicule development has not been studied. Another possibility is

that different signaling pathways interact to generate specificity. Together with the EGF pathway, the Wnt signaling pathway regulates both P11/12 and vulva development as well. We are studying the role of the EGF and Wnt pathways in regulating Hox gene expression in the male tail.

429. Interphylum comparisons of genetic circuits in muscle tissue development

Steven Gregory Kuntz

Understanding the relation of network structure and function in genetic regulatory networks is aided by comparative analysis across different phyla. Striated muscle is an excellent candidate for comparative analysis, being evolutionarily ancient, having striking similarities with pulsating muscle, and being prominent in several model organisms, including mouse (*Mus musculus*) and nematode (*Caenorhabditis elegans*). From extensive genetic and molecular studies, primarily focused on individual components, we know that numerous muscle network components in nematodes, vertebrates, and arthropods are orthologous. It is not known if specific differences in components between the networks perturb the general network structure or function. In order to construct a comprehensive draft map of the striated muscle differentiation networks of mouse and nematode, a computational integration of genetic, expression, and protein-DNA binding data is necessary. Network map comparisons will identify variable and conserved components, as well as similarities and differences among the regulatory properties. This analysis, critical for understanding the relation between the networks, will be used to formulate and test specific hypotheses about the network structure-function relationships. To construct this map for analysis, several techniques will be utilized. To aide in the generation of expression and protein-DNA binding data, a complete genome tiling of *C. elegans* has been designed to allow gene chip analysis of chromatin immunoprecipitation, as well as mRNA expression. RNAi synthetic lethal screens for genetic interactions to aide in the generation of genetic data have been performed. A number of candidate genes identified in the screen are being analyzed for potentially critical roles in muscle tissue differentiation during embryonic development. These genes may help construct the draft map of the regulatory network. With a draft map of the regulatory network, perturbation and cross-species studies of key transcriptional regulators, examined by replacing the *C. elegans* regulators (*unc 120*, *hnd 1*, *hlh 1*, and *hlh 8*) with orthologs from mouse (MEF2A, SRF, HAND, Myogenin, and Twist), will be assayed to learn about the relationship of the orthologous and non-orthologous structure and functionality.

430. *C. elegans* L1 developmental arrest as a model for cancer and aging

Ryan Baugh

Larvae of the nematode *C. elegans* reversibly arrest development in acute response to starvation. Embryos that hatch in the complete absence of food arrest development before any post-embryonic development is observed (L1 arrest), and such arrested worms are resistant to numerous environmental stresses. Arrest can be maintained for weeks and normal development resumed once food is available. In contrast to the well-studied dauer arrest, L1 arrest is near instantaneous and occurs with no morphological modification. I have found that insulin signaling regulates L1 arrest, in addition to dauer arrest and aging. I am using a combination of genetic and microarray analyses to identify other pathways that regulate L1 arrest, as well as genes regulated by insulin signaling during implementation and maintenance of L1 arrest. I am also investigating the coordination of developmental timing with developmental arrest. Proper control of cell division and growth is crucial in preventing cancer, and this system provides a new model to study these controls. In addition, I find it intriguing that individual worms make a coherent all-or-none decision to arrest or develop: While the value of maintaining temporal coordination among the various cell lineages is obvious, the way it is accomplished is unclear.

431. Linker cell migration

Mihoko Kato

The shape of the developing male gonad in *C. elegans* is determined by the migratory path of the linker cell (LC), which is located at the front of the gonad, followed by other somatic gonadal cells and then the germline. While the LC can migrate independently of the rest of the gonad, the rest of the gonad requires attachment to the LC to migrate. I have identified three genes that, when mutated, result in the LC detaching from the gonad and migrating as an individual cell. Based on these genes, I am investigating the potential role of genetic pathways associated with myosin and dynactin protein complexes in this process. I am also using microscopy techniques to look at the cell biology of the LC and the three adjacent cells that directly connect to the LC as this breakdown in cell adhesion and communication occurs.

432. An insect-killing nematode

Elissa Hallem

Heterorhabditis bacteriophora is a nematode parasite of insects that is currently used as a biological control agent for a wide variety of insect agricultural pests. *Heterorhabditis* is symbiotically associated with the pathogenic bacterium *Photorhabdus luminescens*, which colonizes the nematode gut. We found that *Heterorhabditis* can infect *Drosophila melanogaster*, suggesting that the interactions between *Heterorhabditis*, *Photorhabdus*, and *Drosophila* may provide a genetically tractable tripartite model system for the study of insect parasitism and bacterial symbiosis. We are studying the

molecular and cellular mechanisms underlying *Heterorhabditis* infectivity, as well as the effects of *Heterorhabditis* infection on the *Drosophila* immune response.

433. Sensory control of alterations of locomotory behavior during *C. elegans* male mating

Allyson J Whittaker, Gary C Schindelman, Shahla Gharib

We are studying *C. elegans* male mating behavior as a model for understanding how locomotory behavior can be modulated by sensory input. Of particular interest to us are the early steps of male mating behavior, (response, backing and turning), as it is during these steps that the greatest modulation of the normal sinusoidal movement pattern occurs. The hermaphrodite is not thought to play an active role in male mating but instead provide passive cues that trigger different male mating steps. We have been examining the hermaphrodite cue(s) that trigger turning behavior. When we compared the mating behavior of males with hermaphrodites of different lengths, (wild type, lon-2 and dpy-10), we found that males almost always turn within approximately the same percentage body length. This observation suggests that the distance traveled along the hermaphrodite or time the male has spent backing is not important for triggering a turn. To test if a cuticular cue is necessary to signal a male to turn, we have observed the mating behavior of males with bli-6 hermaphrodites. We found that in 51% of encounters with the end of a blister, males initiated a turn, and they fully completed a turn in 20% of encounters. This observation suggests that a specific cuticular cue is not absolutely necessary to trigger a turn. We postulate that it is the change in shape at the head and tail of a hermaphrodite that triggers a male to turn. We are also interested in the neuronal pathways that control response, backing and turning. The effects of mutations in known neuronal signaling genes, as well as mutations isolated in mating defective screens in our lab on these behaviors will be discussed.

434. WormBook: The Online Review of *C. elegans* Biology and Resource for Experimental Methods

Lisa Girard, Tristan Fiedler, Todd Harris, Felicia Carvalho, Igor Antoshechkin, Paul Sternberg, Lincoln Stein, Martin Chalfie

WormBook (http://www.wormbook.org) is a collection of original, peer-reviewed chapters on topics related to the biology of *C. elegans*, as well as WormMethods, a collection of laboratory methods and protocols useful to *C. elegans* researchers. Since WormBook was launched in June 2005 with 12 chapters, it has grown to over 100 chapters, covering nearly every aspect of *C. elegans* research, from cell biology and neurobiology to evolution and ecology, and caters to a wide audience ranging from high school students to established researchers. WormBook is a product of the *C. elegans* community, with nearly 150 of its members already contributing. WormBook also serves as the text companion to WormBase, the *C. elegans* model organism database. In this capacity, WormBook is a paradigm for other model organism database companions. WormBook's electronic publishing model lets it bring information to its readers quickly, and extends its utility with relevant links to other online resources. WormBook content is extensively hyperlinked to WormBase. Genes, proteins, and cells are linked to the relevant pages in WormBase in order to provide our readers with easily accessible background information. Additionally, WormBook contributions contain links to other WormBook contributions, and its in-text citations are linked to their abstracts in PubMed and their full-text version, when available. Since WormBook is online, its chapters are able to contain movies and complex images that would no be possible in print. WormBook is designed to keep up with the rapid pace of discovery in the field of *C. elegans* research and it continues to grow, with the addition of new sections, new chapters, and updated versions of existing chapters.

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Summary: In the Wold group we are interested in the composition, evolution and function of gene regulatory networks, and we often use muscle development, degeneration, and regeneration as a favored model system. We are especially interested in networks that govern how cell fates and states are specified and executed. Our approaches to these problems increasingly use genome-wide and proteome-wide experimental assays coupled with bioinformatic and computational tools needed to extract connectivity relationships and evolutionary relatedness. Much of the informatics side is developed in an on-going partnership with Professor Eric Mjolsness of JPL/University of California, Irvine. In several parts of the work experimental technologies, computational tools, databases, etc., are first developed and tested using yeast as the model genomic system.

An underlying challenge for us is to understand the regulatory events that drive the progression from multipotential precursor cells or adult stem cells to determined unipotential progenitors and then to fully differentiated cells. We are currently studying these cell states and transitions using microarray gene expression analysis, global protein:DNA interaction measures, mass spec-based proteomics of multiprotein complexes, informed by comparative genomics. In mouse and other vertebrates, this lineage arises from paraxial mesoderm to produce muscle (also bone, skin and fat, among other derivatives). Skeletal myogenesis is governed by both positive- and negative-acting regulatory factors. The MyoD family of four closely related, positive-acting transcription factors are key. Upon transfection, each can trigger nonmuscle recipient cells enter, and often to execute, the myogenic differentiation program. Our working model emphasizes that MyoD family factors are able to do this because they are highly connected and cross-talk within their group and with other collaborating factors (like MEF2 factors) and other regulators that now include microRNAs. This drives us to cast our problem in network terms. To understand their extraordinary ability to drive or redirect a cell to become muscle, we need to learn the structure and function of the network(s) in which they are embedded. It is also clear that negative regulators of skeletal myogenesis are probably just as important for regulating the outcome as are the positive regulators. Multiple negative regulators of skeletal muscle are expressed in multipotential mesodermal precursors and in proliferating muscle precursors (myoblasts). It is generally believed that some of these are important for specifying and/or maintaining precursor cells in an undifferentiated state, though exactly how the system works is unknown and a subject for investigation. It has brought us recently to be very interested in defining specific transcriptional repressors and their entire array of *in vivo* target genes. A newer addition asks what role differential translation and RNA-mediated regulatory mechanisms play at each step, and how these might mediate cell communication in the neuromuscular system.

To define protein:protein complexes in the network more comprehensively, we developed a collaborative effort with the Deshaies lab here and the John Yates lab at Scripps to modify and apply MudPIT mass spectrometry, coupled with dual affinity epitope tagging, to characterize multiprotein complexes (Graumann *et al.,* 2004). Among other things, this enabled postdoctoral fellow Libera Berghella to identify a new repressor that acts on myogenin, the MyoD family member most directly responsible for execution of differentiation. New technology development projects are focused on large-scale measurements of protein:DNA interactions, several of which feature a collaboration between our lab and Rick Myers lab and Genome Center at Stanford (see Mortazavi entries below). The computational tools described below have been used to find candidate conserved regulatory elements, and these are, in turn, being subjected to functional assays via lentiviral-mediated transgenesis. These same tools are being used to analyze data from multiple species of worms related, in differing degrees, to *C. elegans.* This is part of an ongoing partnership with the Sternberg lab that involves several joint projects (see Kuntz and Mortazavi entries below). In addition to clarifying how many and which worm genomes give us the most leverage for identifying functionally important noncoding elements in the genome, we seek insights into the evolution ancient gene networks that drive myogenesis and cell cycle across the large phylogenetic distances separating vertebrates, worms, flies and for cell cycle, even plants and yeasts.

Chris Hart's thesis work began with building a data mining and analysis infrastructure called CompClust (Hart *et al*., 2005). He then used it to explore the idea that a simple classifier neural network could be used to integrate complex large-scale data for protein:DNA interactions (chromatin immunoprecipitation assayed by microarray) and global RNA expression data, using multiple sets of yeast cell cycle data. This proved remarkably successful with the neural net models highlighting in the top dozen 10 of 12 previously known major cell cycle transcription factors from datasets for over 200 factors. An interesting and unexpected

implication from his work concerned specific "subnetwork" domains of negative selection imposed on key factor-binding motifs.

Tracy Teal's study of biofilm development is a collaboration with Diane Newman in the Division of Geology and Planetary Science. It has absolutely nothing to do with myogenesis, though it does have to do with a different kind of development. Its thematic goal is to identify, visualize, and ultimately understand the multiple different metabolic cell states that comprise a biofilm at different stages of its development and under differing environmental stimuli. The degree to which principles and regulatory strategies used by metazoans during development are or are not employed by bacteria in creating biofilm structures is being probed by marking bacteria with multiple GFP derivatives driven by genes that will mark functional domains (aerobic vs. anaerobic, for example). To do this, Tracy developed a new and elegant way to characterize biofilm morphogenesis quantitatively. She then used it to establish that the widely held view of mature biofilms having cores composed of dead cells is incorrect, and that these cores are instead in a distinct metabolic and growth state. This growth quiescent but nevertheless live and active state may help to explain some properties of biofilm robustness seen under diverse environmental stimuli, including antibiotic treatment.

435. A new repressor mediates transcriptional down-regulation of myogenin during innervation

*Libera Berghella, Shirley Pease**

Myogenin, one of the four-member MyoD family of bHLH myogenic transcription regulators, is a crucial regulator of myogenesis that is down-regulated at the RNA level during muscle fiber maturation as a consequence of innervation. Comparative genomic analysis of sequences flanking myogenin using an early version of MUSSA (see entry from Tristan De Buysscher below), highlighted a highly conserved 17bp element (myogHCE). We asked what its function is, first by using lentiviral mediated transgenesis. This showed that it mediates and is essential for postnatal repression of myogenin in transgenic animals. Subsequent mass spec analysis of proteins enriched by binding to this motif identified a candidate transacting factor from adult muscle that could be responsible for this regulatory action. It binds myogHCE *in vitro* and occupies the locus in adult skeletal muscle. Altering its levels experimentally shows that it can repress differentiation in C2C12 myoblasts in the gain of function mode, and that it can also regulate multiple effects of innervation in adult muscle. It binds the myogHCE motif as part of a complex that includes the homeodomain protein Pbx. Genome-wide analysis identified a family of related conserved elements, one of which is located 35 Kb upstream of MyoD in mouse and also interacts with MSY-3 *in vivo*. Taken together, these results suggest MSY-3 is a new component of the muscle regulatory network, critical for proper innervation-dependent activity during development.

**Member of the Professional Staff, Caltech*

436. Inferring the structure of the yeast cell cycle transcription network by neural network modeling

Christopher Hart, Eric Mjolsness

A prominent network of kinases (Cdks), together with a coupled system of regulated proteolysis, governs progression of the yeast cell cycle. A major downstream output of this signaling system is transcriptional regulation of a large set of genes, some of which are known to play important roles in further regulating and executing the phases of the yeast cell cycle. Between 300 and 1000 yeast gene RNAs are expressed in a cell cycle-dependent manner. This wide range in the number of genes designated as cycling depends on a combination of experimental specifics, such as the method of phase synchronization, the analytical methods, and criteria for defining cell cycle regulation, but a core of ~200 are common to virtually all studies, and the major kinetic patterns correspond roughly to the phases of the cell cycle. In this project, I used artificial neural networks (ANNs) to integrate results from genome-wide time-resolved RNA expression data from microarrays with large-scale measurements of genome-wide protein:DNA interactions from ChIP-on-chip experiments. The motivation for this method is that this architecture of neural network is very simple, yet captures and capitalizes on several properties of gene networks that are not used by other established methods of analysis. By mining the network weights matrix, 10 of 12 previously known regulatory connections that are associated with the cell cycle by traditional molecular genetics and biochemistry emerged as top connections in the ANN. In addition, several novel connections scored highly by the ANN. These new model-based connections provide a basis for hypotheses about additional regulators acting in the cell cycle. With each of these regulatory relationships we also capture the cell cycle phase in which these regulatory associations are likely to be pertinent.

437. Automation of genome-wide and cross-genome *cis*-regulatory element identification and assessment using Cistematic

Ali Mortazavi, Sarah Aerni[1]

We are continuing development of an extensible computational framework called Cistematic, which is designed to automate discovery and refinement of candidate *cis*-regulatory motifs. It further performs genome-wide phylogenetic mapping for the motif or motif combinations of interest. The objective is to stratify motif occurrences by applying user-specified criteria for phylogenetic conservation and/or site position relative to adjacent coding sequences or other genome features. Motif occurrences that are most conserved are identified using the *cis*Matcher algorithm, which assesses conservation without need of pre-computed multiple-sequence alignments. Resulting sets of conserved motif occurrences, together with identities of the adjacent genes, comprise a "*cis*-motif cohort." A Gene Ontology enrichment module in Cistematic can then be used to test

whether a *cis*-motif cohort is significantly enriched or depleted in members with specific GO functions. This analysis path is especially straightforward and applicable for relatively large-binding motifs such as those typical of many zinc-finger transcription factors.

All data is stored transparently in relational form using the cross-platform Sqlite package. Cistematic provides several layers of abstraction, which allow users of varying levels of sophistication to customize their uses of Cistematic. Thus, while most users will simply supply locus identifiers, and thresholds to the experiment classes and receive standard analysis results, some users may wish to use and extend lower-level objects that handle Motifs, Programs, Genomes, and Homology/Annotation mapping.

We are further expanding Cistematic by adding native implementations of the greedy, Gibbs sampler, and Expectation Maximization motif finding algorithms that form the basis of most *in silico* motif finders used in the field in order to make them available "out of the box." Cistematic currently runs on Mac OS X, Linux, and Solaris, and has a prototype web front-end for users wishing to run Cistematic experiment objects without writing Python scripts.

[1]*Undergraduate, University of California, San Diego, CA*

438. Genome-wide comparative analysis of the NRSF/REST target gene network

Ali Mortazavi

We are investigating the role of a major transcriptional repressor in the evolution of the corresponding vertebrate gene regulatory network (GRN). We are using a combination of computational prediction and direct experimentation to define the genome-wide set of direct targets of the well-known Neuron-Restrictive Silencer Factor (NRSF/REST), which was originally identified as a global repressor of neuronal genes in non-neuronal tissues in work in the Anderson lab at Caltech in the late 80's. It has more recently been shown to repress neuronal genes in stem cells prior to their differentiation. Roles for it have also been suggested in pancreatic development, cardiac lineages, and lymphocyte lineages, although it is not clear whether these roles differ from repression of neuronal genes in non-neuronal tissues during embryogenesis. NRSF is an ideal model factor for defining a regulatory network from the trans-factor point of view, in part because it has a long (21 bp) and relatively well-defined binding motif. Basic network architecture questions include: What are all the *in vivo* targets of NRSF? In what cells are they occupied by the factor and with what consequences? How has this network evolved since its beginnings in the vertebrate lineage?

To approach these initial questions, Cistematic (see Mortazavi and Aerni above) was used across human, mouse and dog genomes to leverage evolutionary conservation to refine the motif model and to locate instances and their adjacent genes. The major experimental tests of sites identified computationally, along with sites of the same size and similar base composition that are predicted not to bind NRSF, are ChIP assays (chromatin immunoprecipitation). Initially ~100 predicted sites from the group of ~700 were assayed by Q-PCR. These allowed us to evaluate the predictive success of our model of the human NRSF target repertoire in Jurkat cells (Mortazavi *et al*., 2006). We are finding similar site occupancy patterns in mouse muscle cells. The composition of the computational target gene set was also interesting because it includes multiple microRNAs and regulators of neuron specific splicing. Among these are microRNAs predicted by sequence to be plausible regulators of NRSF and its Co-Repressor, CoREST, suggesting a dynamic feedback. The perfect conservation of the entire zinc-finger set that comprises the NRSF DNA-binding domain throughout all sequenced vertebrates is striking. Coupled with the absence of any identifiable NRSF transcription factor in all sequenced invertebrates, suggests the notion that the emergence of NRSF in the vertebrates may have been needed to permit "reuse" of neuronal genes in non-neuronal, vertebrate-specific regulatory networks.

To further test and refine the network of true *in vivo* NRSE-binding sites and to begin to assess their functional roles, we are working with Dave Johnson and Evonne Thompson in the Myers group to measure the entire global "NRSFome" experimentally by using microarray and very high throughput sample sequencing (e.g., Solexa, 454, etc.) for ChIP measurements. We are also asking how occupancy of different sites varies with cell type and across species and how they relate to expression pattern and promoter methylation on a genome wide basis.

Planned perturbation experiments will use cells that express NRSF-derived constructs that are, respectively, dominant activating and dominant negative variations of the main isoform, plus the naturally occurring truncated REST4 isoform. Correlates in knockdown or null stem cells can provide supporting data.

439. Interphylum comparisons of genetic circuits in muscle tissue development

Steven Gregory Kuntz

Understanding the relation of network structure and function in genetic regulatory networks is aided by comparative analysis across different phyla. Striated muscle is an excellent candidate for comparative analysis, being evolutionarily ancient, having striking similarities with pulsating muscle, and being prominent in several model organisms, including mouse (*Mus musculus*) and nematode (*Caenorhabditis elegans*). From extensive genetic and molecular studies, primarily focused on individual components, we know that numerous muscle network components in nematodes, vertebrates, and arthropods are orthologous. It is not known if specific differences in components between the networks perturb the general network structure or function. In order to construct a comprehensive draft map of the striated muscle differentiation networks of mouse and nematode, a computational integration of genetic, expression, and protein-DNA binding data is necessary. Network map

comparisons will identify variable and conserved components, as well as similarities and differences among the regulatory properties. This analysis, critical for understanding the relation between the networks, will be used to formulate and test specific hypotheses about the network structure-function relationships. To construct this map for analysis, several techniques will be utilized. Preliminary analyses of *cis*-regulatory elements of the Hox gene cluster of *lin 39* and *ceh 13* in *C. elegans* has demonstrated the utility of computational techniques in efficiently identifying relevant elements for analysis and will aid in the identification of network components. To aide in the generation of expression and protein-DNA binding data, a complete genome tiling of *C. elegans* is being designed to allow ChIP on chip, as well as expression on chip analyses. RNAi synthetic lethal screens for genetic interactions will aide in the generation of genetic data. With a draft map of the regulatory network, perturbation and cross-species studies of key transcriptional regulators, examined by replacing the *C. elegans* regulators (*unc 120*, *hnd 1*, *hlh 1*, and *hlh 8*) with orthologs from mouse (MEF2A, SRF, HAND, Myogenin, and Twist), will be assayed to learn about the relationship of the orthologous and non-orthologous structure and functionality.

440. A general cohybridization standard for microarray gene expression measurements using amino-allyl-labeled genomic DNA

Richele M. Gwirtz, Brian A. Williams

Microarray gene expression data is best exploited when results can be compared and analyzed across many experiments within and between laboratories. This requires optimization and adoption of a standard set of experimental techniques. Variability in the mass of hybridizable material deposited on the features of a microarray is a source of systematic error which must be controlled before meaningful comparisons between experiments can be made. This is typically done using ratiometric quantitation, in which the labeled cDNA sample is cohybridized to the array in the presence of a differently labeled reference standard. Although ratiometric quantitation is a widely used method for gene expression measurement on spotted microarrays, there is no consensus about the appropriate material to be used as a reference standard. An ideal standard should be relatively inexpensive and easy to prepare, should provide coverage of all array features at equimolar intensity, and should be biologically invariant and technically easy to reproduce. We have developed and now refined the simplest and most comprehensive possibility, which is genomic DNA. In this capacity, genomic DNA provides equimolar coverage of microarray features [Williams *et al*. (2004) *Nucl. Acids Res.;* Williams *et al*. (2006)]. Equimolar representation allows for the direct comparison of abundance levels between features on the same array, because it preserves the prevalence proportions of individual RNA transcripts present in the experimental sample.

We improved the convenience and ease of use by adapting an indirect labeling method that allows one to make and characterize a large batch of labeled genomic DNA standard that can be stored and drawn on over many experiments. This indirect labeling method uses amino-allyl dUTP, followed by chemical coupling of the amino-allyl group to a fluorescent moiety. Our data show that this effectively addresses the problems of variation in labeling efficiency that result from the direct incorporation of fluorescently-labeled nucleotides. It also reduces experimental risk, since large quantities of the standard can be prepared in advance and quality tested prior to committing precious cDNA samples and microarrays to hybridization. Comparison of intensity measurements between positive hybridization probes (true positives) and negative control probes (true negatives) shows that this method reliably separates their fluorescent intensities under standard hybridization and scanning conditions. The proportion of array features covered by the genomic DNA standard is greater than that covered by commercially available alternatives, and feature intensity is more uniform.

441. Microfluidic chaotic mixing devices improve signal to noise ratio in microarray experiments

Jian Liu, Brian Williams, Richele Gwirtz, Barbara Wold, Stephen Quake

In conventional microarray experiments, hybridization of labeled target DNA molecules to sequence-specific probes relies on diffusion in a static hybridization solution volume. During the time interval for a conventional microarray experiment, a target molecule can be expected to traverse a distance of about 1-3 mm. The dimensions of a typical microarray are >10 mm, therefore, a large portion of the available labeled target does not encounter its cognate sequence probe for specific hybridization. We have attempted to improve the performance of microarray hybridization using a microfluidic chaotic mixing chamber to perform dynamic hybridization. We fabricated a two-layer polydimethylsilicone (PDMS) microfluidic mixing/hybridization chamber and sealed it to a spotted microarray slide. The fluidic layer of the device contains two symmetric hybridization chambers (6.0 mm x 6.5 mm X 65 microns). They are connected to each other by bridge channels, whose ceiling contains a series of indentations in a herring-bone pattern, which produces chaotic mixing of the hybridization solution. Four input/output through-holes with corresponding micromechanical valves are used for loading sample solutions or disposing waste buffers. The valves are actuated to form closed chambers during the circulation of the fluid. Additionally, in the control layer two sets of peristaltic pumps are integrated to move the fluid between the hybridization chambers.

Test hybridizations were performed on spotted microarrays containing 4321 gene-sequence-specific 70 mer oligonucleotide probes, with an additional 65 randomized sequence 70 mer probes as negative controls.

Both static and dynamic hybridizations were performed for a 2 hr time interval, and hybridization intensities for positive probes and negative controls were compared. Receiver-operating characteristic (ROC) curves for the two experiments indicate a much greater separation in the distribution of positive probe intensities compared to negative control intensities for the dynamic hybridization experiment than for the static experiment (area under the ROC curve for dynamic hybridization = 0.92; area for static hybridization = 0.73). Experiments are in progress to test the performance of these methods at longer hybridization intervals. The dynamic mixing method has the potential to increase the detection sensitivity for conventional spotted microarrays at a relatively low cost. It is compatible with commercially available microarray slides, and offers the possibility of integration into large-scale nucleic acid sample processing operations.

442. Spatiometabolic stratification of *Shewanella oneidensis* biofilms

*Tracy Teal, Dianne Newman**

Biofilms, or surface-attached microbial communities, are both ubiquitous and resilient in the environment. It is now recognized that many bacteria in the environment grow as surface-attached microbial biofilms, and it has been suggested that this biofilm lifestyle may account for the remarkable persistence of bacterial populations in the face of changing environmental conditions. Biofilms are composed of many hundreds to millions of cells, each of which experiences its own microenvironment due to strong chemical gradients that are established by metabolism and diffusion. Biofilms are therefore heterogeneous and spatially stratified, so that activity levels and biochemical processes occur differentially according to the location of a cell in the biofilm and the biofilm structure's biomass.

Although much is now known about how biofilms form, develop and detach, very little is understood about how these events are related to metabolism and its dynamics. It is commonly thought that large sub-populations of cells within biofilms are not actively producing proteins or generating energy, and are therefore dead. Early results in this project led us to hypothesize that within the growth-inactive domains of biofilms, significant populations of living cells persist and that they retain the capacity to dynamically regulate their metabolism. To test this, we employed unstable fluorescent reporters to measure growth activity and protein synthesis *in vivo* over the course of biofilm development and created a quantitative routine to compare domains of activity among independently-grown biofilms. We found that *S. oneidensis* biofilm structures do indeed reproducibly stratify with respect to growth activity and metabolism as a function of their size. Moreover, within domains of growth-inactive cells, genes that are typically upregulated under anaerobic conditions are expressed well after growth has ceased. These findings show that, far from being dead, the majority of cells in mature *S. oneidensis* biofilms have actively turned on metabolic programs appropriate to their local microenvironment and developmental stage (Teal *et al*., 2006). This raises a new set of questions concerning the relationship of these cells in a biofilm to the stationary phase of cells growing in planktonic culture; the full range of metabolic activities performed by these cells; and the nature of signaling systems that lead to defining this state.

**Professor of Geobiology and Biology, Caltech*

443. Role of micro RNA species during cell type differentiation

Brian Williams, Richele Gwirtz, Gilberto DeSalvo

Specific gene activation and gene silencing mechanisms play important roles during cell state transitions that lead to cellular differentiation, including the myogenic pathway. At least two aspects of the problem are likely to involve small regulatory RNAs. One is transcriptional repression, where we are especially intrigued by the possibility that a number of zinc-finger regulators may operate either entirely or partly, in repressing modes. Micro RNAs are 18-25 nucleotide sequences produced from longer RNA hairpin precursor molecules. Although studies of micro RNAs in mammalian systems have lagged behind plant, worm and fly systems, it is increasingly clear that they function in two major modes in all systems to effect gene silencing. Post-transcriptional gene silencing (PTGS) is effected by either destruction of extant messenger RNAs or by control of translation. Transcriptional gene silencing (TGS) involves recruitment of histones and the DNA methylation apparatus to a genetic locus, thereby preventing initiation of transcription. Still other modes of small RNA regulation are beginning to emerge whose mechanism of action and importance are less certain. Globally, we are exploring the role of major repressing systems in the myogenic pathway. This branch of the exploration focuses on small RNAs and their relationship to representatives of three major types of zinc-finger regulators that are known at present for their direct association with DNA-binding sites where they recruit co-repressors and effect shutdown of nearby target genes (the rapidly evolving Krab repressor family; the multipurpose transcriptional activator/silencer/insulator called CTCF; and the neuronal repressor NRSF (see entry from Mortazavi). We reasoned that, since a classic zinc-finger transcription factor (TFIIA) has long been known to interact specifically with both DNA and RNA targets, this duality may be much more widespread, and we are testing this idea. We are interested in the degree to which forms of TGS, which is far better documented in plants, may be operating as part of a major modulatory network in vertebrate systems. The recent, but still poorly understood, observation that a small dsRNA version of the binding site for NRSF, is expressed in neural stem cells where it may modulate action of the repressor – or even turn it into an activator – lends impetus to the exploration [Kuwabara (2004) *Cell* 116:779].

Western blot analysis of protein isolated during a time course of skeletal muscle differentiation in the C2C12 mouse skeletal muscle cell line initially focused on four

factors or cofactors that might engage small RNA substrates in a specific manor: CTCF, a repressor protein with wide-ranging functions; KAP1, a protein associated with the Krüppel-associated box (KRAB) zinc-finger protein; NRSF; and Ago4, a member of the Argonaute class of PAX/PIWI domain proteins that aids in the colocalization of microRNAs with the microRNA processing machinery. We find that all four are present in C2C12 cells, and exhibit kinetics of expression that correlate (or anti-correlate) with the progress of differentiation. We are using co-immunoprecipitation experiments from nuclear fractions of C2C12 cells during differentiation, to recover microRNAs with which they may be specifically associated. In a second related group of experiments we are testing for the presence and expression pattern of known and hypothesized microRNAs that correspond to recognition sequences of interest for these regulators.

444. Transcriptional regulation of genes underlying skeletal muscle atrophy

Gilberto Hernandez, Jr.

My goal is to identify and then learn the function of components of the transcriptional network controlling muscle atrophy genes. A major contributor to the profound stereotypic proteolysis seen in skeletal muscle atrophy is the ubiquitin proteosome pathway, and two key E3 ubiquitin ligases mediate much of this effect. They are Muscle Atrophy F-box (MAFbx) and Muscle RING Finger-1(MuRF-1). Current understanding of the signaling cascade(s) regulating muscle mass have already benefited from the use of MAFbx and MuRF-1 as primary regulatory targets and molecular effectors of atrophy. However, the explicit transcriptional regulatory network controlling MAFbx and MuRf-1 remains largely unknown. By working from functional *cis*-regulatory elements, identified partly by their conservation through evolution and partly by experiment, we aim to define components of the transcriptional network underlying skeletal muscle atrophy. In addition, we hope to use these *cis*-acting regulatory regions, and sequence motifs within them, to identify other genes that share them and are part of the atrophy response gene network.

Using comparative genomic sequence analysis, I made a catalog of non-coding, putative *cis*-regulatory elements for both Mafbx and Murf-1 genes and established an *in vitro* atrophy assay, which recapitulates the initial suppression and activation of these genes. Sequence similarity analysis and a focus on shared motifs within 3'UTR regions has led a focus on specific elements as predicted target sites for transcription factors binding or for microRNA interaction. These are presently being tested in more detailed transfection assays to establish which elements, or combinations of elements, are needed to regulate expression of Mafbx and Murf-1.

445. CompClust Web, a data analysis tool for array datasets

Diane Trout, Brandon King, Joe Roden

The CompClust environment is a Python computational framework for mining and analyzing large-scale biological dataset. We initially designed it for processing various types of microarray data types such as: two-color, Affymetrix, and ChIP-chip. However, many of the tools will also be applicable to other forms of large-scale data. The essential architecture point is that CompClust provides the ability to attach annotation labels to arbitrary slices of the array, which are then maintained throughout all subsequent transformations and plots CompClust is capable of.

Current effort is focused on extending the utility to a wider group of biologists by developing a web-based user interface that rests on top of the original Python interpreter environment and provides access for the less computationally oriented. Once a dataset is loaded and properly annotated CompClust provides rich set of clustering algorithms and principal component analysis to tease apart meaning hidden within the data. The clustering tools include KMeans, KMedains, EM Diagonal Mixture of Gaussian, as well as various cross validation algorithms designed to perform arbitrary parameter searches. One of the strengths of CompClust is the set of tools it provides to compare various clusterings through the use of both visual tools like the confusion matrix and quantitative scores like normalized mutual information and linear assignment. Individual clusters can also be explored with the receiver operator curve tool that shows the overlap of a cluster with its neighboring data points[1].

We recently added a significant extension of the previous principal component analysis that enables automatically identifying sets of biologically relevant genes through a combination of principal component analysis and information theoretic metrics (Roden *et al.*, 2006). It permits a user to visualize and mine each of the independent sources of significant variation present in gene array datasets in order to identify the genes most affected by specific groups of conditions or tissues and to suggest what underlying factors may be driving the variation. More information about CompClust is available from http://woldlab.caltech.edu/compclust.

In addition to the binary builds for Windows and source distributions for Linux and OS X, we also have a variety of documentation and tutorials available.

Reference

1. Hart, C.E., Sharenbroich, L., Bornstein, B.J., Trout, D., King, B., Mjolsness, E. and Wold, B.J. (2005) *Nucl. Acids Res.* **33**(8):2580-2594.

446. BioHub

Brandon King, Joe Roden

The goal of the BioHub database project is two-fold. The first is to provide our biologists with a tool that allows them to ask questions of very different kinds of large-scale biological data which are tied together based on their spatial relationship to a gene or DNA sequence feature in one or more genomes. The computational goal is to make a rich API (Application Programming Interface) to allow computer scientists to easily write custom large-scale analysis programs, which can then be turned into web application or other GUI to allow for easy to use large-scale analysis.

In it's current form, BioHub is a spatial annotation PostgreSQL database with a Python API for writing applications. It works by registering sequences (annotations of sequences) in the BioHub core database. Upon registering an annotation at a location on a genome, the user or program receives an SID (Sequence ID) that can later be used as a handle to the 'Registered Sequence' when using the BioHub API. An SID will always be the same for the exact same location on a genome. This means that if two different programs or people register a sequence with the exact same location, both will be given the exact same SID. This feature is important because it allows for connection of a wide variety of biological data to associated by simply having the same location on the genome. For example, if one were to register a sequence they found in a publication as a 'conserved regulatory motif' and then later a motif finding program finds the exact same motif, they will both have the exact same SID. But they will also have two descriptions and users attached to the SID, as well. One saying "found in paper x..." and the other "discovered by motif finding program y." By simply registering the two sequences, the published motif has now been connected to all sites in BioHub, and in current work the hub is expanding to allow the next obvious query to recover all expression data associated with this custom set of instances. The user has the capacity, through BioHub, to specify and collect, via SIDs, only those genes associated with motif instances that have a particular positional relationship to your gene models. BioHub was used to design a custom gene chip that discriminates hundreds of related zinc-finger transcription factors in the human genome. These are not well represented with non-crossreacting probes in current commercial array collections.

447. Comparative genome analysis over more than three genomes using MUSSA and MUSSAGL

Tristan De Buysscher, Diane Trout

Comparative genome analysis, as a routine lab tool for cell and molecular biologists, is becoming increasingly important as the number of fully sequenced genomes increases. In general, the more genomes included in an integrated comparative analysis, the better the resolution of functional elements conserved due to selection, versus sequences that are the same by chance. Great evolutionary pressure in such a comparison can also be exerted by identifying the very rare elements that are recognizably similar over a single very long distance, such as human to fish. However, we find that this approach, as would be expected, is very severe and has the effect of eliminating many known functional enhancers and promoters that we want to be able to detect. The availability of many mammalian genome sequences means that we can get evolutionary leverage by integrating over larger numbers of genomes each of which is individually not so distant from the reference mouse or human genome. Once these basic comparisons are made we want to relate domains of sequence conservation to each other, ask if they have shared internal elements, and map additional features such as small transcription factor-binding motifs, regulatory RNA interaction motifs, etc. MUSSA (multiple sequence similarity assignment software) is an interactive viewer that was designed to do analysis of this kind, at the level ~100 kb regions for N genomes, rather than for two or three. It uses a transitivity-filtering algorithm to integrate sequence similarities over the entire collection of genomes being compared. The underlying sequence similarity mapping was done with a classic sliding window method, implemented as it was for the earlier FR project with the Eric Davidson lab. MUSSA interactivity features permit inspection of the analysis at varying levels of resolution, recovery of specific sequence regions for further external analysis, and user-driven integration of conserved sequence domains with maps of sequence motifs such as transcription factor-binding sites or gene model annotations. MUSSA analysis has been done on multiple worm genome sequences with Erich Schwarz and Paul Sternberg and Steven Kuntz, and on several genes of interest in the myogenic regulatory network from varying numbers of mammalian genomes. These comparisons highlight both known and new candidate sequence domains and candidate factor-binding motifs within those domains. Experimental tests for regulatory function in transgenic assays are underway and a majority of noncoding conserved elements from the lin39-ceh13 Hox locus are proving to be active. MUSSA is available from the Wold website.

448. A PCA-based way to mine large microarray datasets

Joe Roden, Chris Hart, Brandon King, Diane Trout

In many instances where large-scale microarray analysis is part of a project, the biologically important goal is to identify relatively small sets of genes that share coherent expression across only some conditions, rather than all or most conditions, which is what traditional clustering algorithms find. The PCA (principle components analysis)-based tool developed in this project performs a complementary kind of datamining that helps one identify groups of genes that are highly upregulated and/or down-regulated similarly, across only a subset of conditions. Equally important is the need to learn which conditions are the decisive ones in forming such gene sets of interest, and how they relate to diverse conditional

covariates, such as disease diagnosis or prognosis. This is a nontraditional use of PCA, and the software tools for doing it will be accessible from the group website. The method is described in a recent paper (Roden *et al*., 2006) and the software is openly available at the Wold group website.

Publications

Hart, C.E., Sharenbroich, L., Bornstein, B.J., Trout, D., King, B., Mjolsness, E. and Wold, B.J. (2005) A mathematical and computational framework for quantitative comparison and integration of large-scale gene expression data. *Nucl. Acids Res.* **33**(8):2580-2594.

Hart C.E., Mjolsness, E. and Wold, B.J. (2006) Connectivity in the yeast cell cycle transcription network: Inferences from neural networks. *PLoS Computation* **3**.

Liu, J., Williams, B.A., Gwirtz, R.M., Wold, B.J. and Quake, S. (2006) Enhanced signals and fast nucleic acid hybridization by microfluidic chaotic mixing. *Angew Chem. Int. Ed. Engl*. **45**:3618-3623.

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Teal, T.K., Lies, D.P., Wold, B.J. and Newman, D.K. (2006) Spatiometabolic stratification of *Shewanella oneidensis* biofilms. *Appl. Environ. Microbiol.*

Williams, B.A., Gwirtz, R.M. and Wold, B.J. (2006) Genomic DNA as a general cohybridization standard for ratiometric microarrays. *Methods Enzymol.* **410**:237-279.

Facilities

Flow Cytometry and Cell Sorting Facility
Genetically Altered Mouse Production Facility
Millard and Muriel Jacobs Genetics and Genomics Laboratory
Monoclonal Antibody Facility
Nucleic Acid and Protein Sequence Analysis Computing Facility
Protein Expression Center
Protein Microanalytical Laboratory

Flow Cytometry and Cell Sorting Facility
Supervising Faculty Member: Ellen Rothenberg
Facility Manager: Rochelle Diamond
Operators/Technical Specialists: Stephanie Adams, Rochelle Diamond, Patrick Koen, Diana Perez

The Caltech Flow Cytometry Cell Sorting Facility is a multi-user facility that has been providing flow cytometric technology on a fee-for-service basis to the Caltech community for over 20 years. The power of flow cytometry is its ability to analyze, at high speed, light scatter and fluorescent properties of heterogeneous populations of suspended particles such as cells, micro-organisms, and/or beads. These measurements are correlated on a single-particle basis to yield both qualitative and quantitative information, and statistical population characteristics are revealed for all components of the sample. In addition, the particles can be sterilely sorted based on their individual characteristics into tubes or multi-well plates of several configurations. Because fluorescence can be used as a quantitative indicator of many different molecular and cellular properties, this technology is a multifaceted tool that can be used to study many aspects of cell biology including cell cycle, physiological function, and membrane antigen phenotype. It has direct use in immunological, neurobiological, and developmental systems to define and isolate cell populations of interest. Some examples are: isolation of transfectants that exhibit reporter gene expression that can be correlated with other desired properties; real-time measurements of physiological responses in cells such as ion usage; quantitation of cell death; isolation of populations for preparation of cDNA for qPCR and gene chip analyses; and single-cell cloning by direct cell deposition.

This Facility is different from most cell sorting facilities in medical schools throughout the United States because of the unconventionality of the applications it supports. A relatively small fraction of the Caltech user groups employ the facility for separation of immune system cells, which is by far the most common use of flow cytometric technology. These currently include the Baltimore, Patterson, and Rothenberg groups. Other users focus on nonmammalian or nonhematopoietic cells, and/or the fluorescent detection of novel compounds and biological indicators. For example, the David Anderson lab uses the facility regularly for mammalian cell work, but for developmental neurobiology and endothelial patterning studies. The Elowitz lab uses the facility to select bacteria with heritable variability in gene expression; the Smolke lab uses it to study inducible promoter networks and control of RNA splicing in yeast. The Campbell group uses the facility to determine the roles of genes in particular aspects of the yeast cell cycle. The Tirrell lab is using the facility to develop methods for imaging newly synthesized proteins in mammalian cells, and to select *E. coli* expressing tRNAs that accept non-natural amino acids. A collaborator at USC, Dr. Bottini, has used the facility to screen fluorescent probes for *in vivo* activity of particular protein tyrosine phosphatases. The Sessions group has used the facility to isolate bacteria and eukaryotic microbes from environmental samples for isotopic composition analysis. The Dervan group has used the facility to measure functional impacts of DNA-targeting pyrrole-imidazole polyamide-chlorambucil compounds, while the Barton group is using it to study uptake of ruthenium(II) dipyridophenazine into cells and nuclei. Many of these unconventional applications have required special system modifications or *de novo* protocol design, which have been aided through the unique consultation and application development services that our facility provides.

The facility is well equipped with two sorters, two analyzers, and a workstation. Our newest sorter, the BD FACSAria, is an industry-leading instrument that analyzes over ten colors of fluorescence with all-digital signal processing and sorts up to four populations in parallel at high speed with minimal phototoxic damage to the cells. The FACSVantage SE is a consistent full-featured sorter that can sort up to 3000 cells per second and detect up to six colors. An older, non-sorting Beckman Coulter Elite that can analyze up to three colors is excellent for cell cycle work. The non-sorting BD FACSCalibur can analyze four colors and is available to researchers around the clock, provided that the investigators have demonstrated competence with the analyzer or take training provided by the facility. In addition the facility provides a 10 client site license for Treestar's FlowJo off-line analysis for users of the facility**.**

This past year, the facility has serviced 23 research groups spanning over 50 researchers from the Biology, Chemistry, Chemical Engineering, and Geobiology Divisions. Facility operator Stephanie Adams left in spring of 2006 to enroll in a prestigious Ph.D. program, and Diana Perez has capably stepped into her place.

Publications in the past year that were based on work done in the Facility include the following:

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Genetically Engineered Mouse Services
Director and Member of Professional Staff: Shirley Pease
Cryopreservation and Microinjection: Juan Silva, B.S., LAT
Embryonic Stem Cell Culture: Jue Jade Wang, M.S.
Mouse Colony Manager: Jennifer Alex, A.A., RLATG

In June 2005, the Genetically Altered Mouse Core and the Office of Laboratory Animal Resources, (OLAR) combined to form the Caltech Laboratory Animal Services, (CLAS). CLAS consists of two subdivisions, OLAR, which is headed by Dr. Janet Baer and Genetically Engineered Mouse Services (GEMs) that is headed by Shirley Pease. The purpose of the merger was to refine, streamline and standardize procedures for laboratory animal care and use on campus. GEMs continues to provide microinjection, cryo-preservation, re-derivation and tissue culture services. In addition, we offer services in the form of rodent colony management and use, where required, in all animal Facilities.

Gene addition in the mammalian system is accomplished by injecting DNA into the pronucleus of a fertilized egg (Gordon *et al*, 1980). This is a non-targeted event. Targeted disruption of specific genes, however, requires the manipulation of pluripotent embryonic stem (ES) cells *in vitro* and their subsequent return to the embryonic environment for incorporation into the developing embryo (Zijlstra *et al*, 1989). The resulting chimeric mouse born is useful for two purposes: 1) it is comprised of tissue from two sources, the host embryo and the manipulated stem cells. More importantly, 2) it can be mated to produce descendants that are entirely transgenic, resulting from the ES cell contribution to the germline of the chimeric mouse. The facility, in collaboration with Anderson, Baltimore, Kennedy, Lester, Patterson, Rothenberg, Simon, Varshavsky and Wold laboratories, has generated multiple transgenic, knockout and knockin mouse strains, amounting to nearly 160 mouse strains. More recently, the Facility together with the Baltimore lab, participated in the development of a new method for the introduction of DNA into early-stage embryos (Lois, *et al*., 2002). This method makes use of non-recombinant lentivirus as a vector for the introduction of DNA into one-cell embryos. The method has proven to be highly efficient and promises to be useful for studies in mice and rats, where large numbers of constructs need to be tested. This new methodology also makes feasible the generation of transgenic animals in species that were hitherto impractical to work with, due to the very low numbers of embryos available for use. Since the lentiviral vector method was established, 79 transient or established mouse models have been generated by this means, together with one Tg rat model. Facility staff has performed all embryo manipulation involved in the production of these new lines.

Microinjection equipment has been set up within the mouse facility, which operates on restricted access as part of a barrier system designed to safeguard the microbiological status of the animals. A room outside the facility has been allocated by the Division to be used primarily for teaching graduate students, technicians and postdocs the techniques involved in transgenic mouse production. This room has been operating since July 1996. Investigators have the option of using this room to perform their own microinjection of embryos, rather than using the full technical service available from the Genetically Engineered Mouse services.

At the merger, a new position was created, that of Colony Manager. An experienced technician has been appointed to the post and is available now to assist investigators with all colony management questions, primarily assisting investigators in making sure their experimental needs are met as economically as possible.

In tissue culture and the use of embryonic stem (ES) cells, the Facility participated in the derivation of new ES cell lines derived from genetically altered mice (see Simon laboratory Annual Report, 2001). Several investigators are using these pluripotent cells in research that involves pushing the cells down specific developmental pathways, and also to investigate the incorporation of extraordinarily large pieces of DNA into the mouse genome. Last year, we acquired several new embryonic stem cell lines, including a hybrid cell line, and two C57BL/6 derived lines. We have tested the efficiency of one of the C57BL/6 ES cells lines and find that we can obtain germline transmission with these cells after electroporation and selection. Before the end of the year, we will electroporate our two C57BL/6 ES cell lines side by side, to determine which will give the best results, before making these cells available to all investigators. C57BL/6 ES cells provide a significant advantage in that the mutation will be established initially on this well understood genetic background, instead of undertaking a two-year breeding program to reach the same point, having initially established the mutation on a sub-optimal genetic background. Hybrid ES cells may be useful for their reported vigor.

Once a new mouse model has been characterized, it may be cryopreserved, or sent to the Mutant Mouse Resource Center, to be made available to the research community in general. We currently have 84 mouse models cryopreserved. For each line, between 200 and 500 embryos at 8-cell stage have been preserved in liquid nitrogen. There are currently 26,033 embryos frozen in total. We shall continue to preserve embryos from mouse strains. The advantages of such a resource are many. Unique and valuable mouse strains that are currently not in use may be stored economically. In the event that genetic drift should affect any strain, over time, then the option to return to the original documented genetic material is available. Lastly, in the event of a microbiological or genetic contamination occurring within the mouse facility, we have the resources to set up clean and genetically reliable mouse stocks in an alternative location.

In the coming year, Facility staff will be receiving training on the culture of human embryonic stem cells.

Presently, eleven principal investigators and their postdoctoral fellows or graduate students use GEMs services. In addition to the maintenance of nearly 100 different targeted and non-targeted strains, we also maintain colonies of inbred and outbred animals, which are used to support the development of new lines, by investigators at Caltech. We also have many mouse models on both an inbred and an outbred background, plus intercrosses between two or three different but related mouse models. In total, we currently maintain nearly 200 separate strains of mouse. Some of these strains are immune deficient and require specialized care, although not pathogenic, to protect them from bacteria commonly present in immune-competent animals. In immune-deficient animals, these hitherto harmless organisms can cause a problem. This may interfere with the well being of the animal and the extraction of reliable experimental results.

Listed below are the names of the eleven principal investigators and their postdoctoral fellows or graduate students who are presently using the transgenic facility.

David Anderson
Gloria Choi, Ben Deneen, Wulf Haubensak, Christian Hochstim, Walter Lerchner, Li Ching Lo, Agnes Lukaszewic, Sophia Vrontu.

David Baltimore
Shengli Hao, Lili Yang

Mark Davis
Derek Bartlett, Eric Kowel

Scott Fraser
Carol Readhead

Mary Kennedy
Holly Carlisle, Eduardo Marcora, Andrew Medina-Marino, Leslie Schenker, Laurie Washburn

Henry Lester
Purnima Deshpande, Carlos Fonck, Princess Imoukhuede, Herwig Just, Raad Nashmi

Paul Patterson
Ben Deverman, Ali Koshnan, Natalia Malkova, Limin Shi, Stephen Smith

Ellen Rothenberg
Deirdre Scripture-Adams, Chase Tydell, Mary Yui, Mark Zarnegar

Melvin Simon
Valeria Mancino

Alexander Varshavsky
Christopher Brower, Jun Sheng, Jianmin Zhou

Barbara Wold
Richel Gwirtz, Brian Williams

Publication

Pease, S. (2006) Ancillary Techniques. In: *Mammalian and Avian Transgenesis New Approaches*. S. Pease and C. Lois (Eds), Springer of Berlin, Heidelberg and New York, pp. 231-276.

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Millard and Muriel Jacobs Genetics and Genomics Laboratory

Director: José Luis Riechmann
Staff: Brandon King, Vijaya Rao, Lorian Schaeffer, Erika Strandberg, Joanne Tan-Cabugao

Support: The work described in the following reports has been supported by:

Muriel and Millard Jacobs Fund for Genomics and Genetic Technology
Millard and Muriel Jacobs Family Foundation
National Science Foundation

Summary: The goal of the Millard and Muriel Jacobs Genetics and Genomics Laboratory, in the Division of Biology, is to provide a suite of cutting edge genomic research tools to all interested Caltech scientists, with an initial emphasis on large-scale gene expression profiling. The Laboratory performs gene expression analyses using DNA microarray technology, and is equipped with the necessary experimental and bioinformatics infrastructure that is needed to generate, store, and analyze large-scale datasets from a variety of microarray technological platforms.

In addition to the broad mission of the Laboratory, we are interested in the analysis of regulatory networks in *Arabidopsis* using genomic technologies, in particular those networks that are related to flower development. An important, but only relatively recently characterized, class of regulatory molecules in animals and plants are microRNAs (miRNAs): small non-coding RNAs (~20-24 nt in length) that regulate gene expression in a sequence-specific manner by targeting mRNAs for cleavage or translational repression. We are using genomic technologies (such as DNA microarrays) to characterize the *Arabidopsis* complement of microRNAs and its participation in floral development processes.

Infrastructure and capabilities:

The laboratory is equipped with the instrumentation necessary to manufacture microarrays and to use commercial Affymetrix GeneChips. Available equipment in the laboratory includes a MicroGrid II arrayer (BioRobotics), a GenePix 4200A scanner (Molecular Devices/Axon Instruments), a BioAnalyzer (Agilent Technologies), a QIAGEN 3000 liquid handling robot, and an Affymetrix GeneArray 3000 7G scanner and fluidics station. A high-throughput real-time PCR system (LightCycler 480, from Roche) has recently been added to the laboratory. A more comprehensive description of the laboratory infrastructure is available at: http://mmjggl.caltech.edu/.

The MicroGrid II arrayer allows us to produce large numbers of microarray slides in a cost effective manner, and to produce customized microarrays for which there is no equivalent commercial counterpart available. For example, we produce whole genome *Arabidopsis* microarrays using 70-mer oligonucleotide sets from Operon, which are used in various research projects related to flower development (work performed together with Professor E.M. Meyerowitz's group). Custom carbohydrate microarrays are used to study glycosaminoglycan-protein interactions (Professor Linda Hsieh-Wilson, Division of Chemistry). MicroRNA microarrays have been produced for mouse (Professor David Baltimore) and *Arabidopsis* (see below). Other customized microarrays include yeast intergenic chips (Professor Judith Campbell), and microarrays printed with Cy3-labeled probes, which are used to study the dynamics of DNA hybridization in real time (Professor Babak Hassibi, Division of Engineering and Applied Science).

The laboratory is equipped to use the latest generation of Affymetrix GeneChips. Among the research groups at Caltech that have benefited from that technology this past year are those of Professors David Anderson, David Baltimore, Seymour Benzer, Marianne Bronner-Fraser, Peter Dervan, Henry Lester, Paul Patterson, Ellen Rothenberg, and Alex Varshavsky.

The laboratory uses Resolver (from Rosetta Biosoftware) as its primary gene expression data analysis system. Resolver is a robust, enterprise-scale, gene expression system that combines a high capacity, MAGE-compliant database and advanced analysis software in a high-performance server framework. The system is accessible through client stations using a web-based interface. We also have at our disposal additional microarray software tools and analysis packages, both public and commercial. The hardware infrastructure of the laboratory currently includes a Sun Fire V880 server (from Sun Microsystems), that we use for the Resolver database (Oracle) and analysis system.

449. Genomic analyses *Arabidopsis* miRNAs: Their roles in flower development

Brandon King, Vijaya Rao, Lorian Schaeffer, Patrick Sieber[1], Joanne Tan Cabugao, José Luis Riechmann

The focus of this project is to characterize the functions of the *Arabidopsis* complement of microRNAs (miRNAs), and to identify the gene regulatory networks in which they may participate - in particular during flower development, by using microarray analysis of miRNA expression and other genomic approaches. MicroRNAs are small non-coding RNAs that regulate gene expression in a sequence-specific manner, and they have emerged as a very important class of regulatory molecules in plants and in metazoans. In *Arabidopsis*, more than 100 miRNAs have already been detected and/or predicted, and the *Arabidopsis* genome may in fact contain several hundred distinct miRNA loci - a number that demands the development of high-throughput methodologies for their study.

We are revising, and extending, the computational identification of *Arabidopsis* microRNAs. We are also developing a microarray platform for miRNA expression detection, which will be used (together with our standard gene expression microarrays) to conduct miRNA expression profiling experiments aimed at understanding

the roles of miRNAs during flower development, at gaining experimental evidence for predicted miRNAs, and at identifying miRNA targets. We have initiated the first round of experiments on miRNA expression profiling by growing the first cohorts of plants and purifying samples of small RNAs, and we are preparing a variety of additional *Arabidopsis* transgenic and mutant lines.

[1]*Postdoctoral Scholar, Division of Biology, Caltech (Meyerowitz laboratory)*

Publications

Riechmann, J.L. Transcription factors. In: *Regulation of Transcription in Plants*, K.D. Grasser (ed.), Blackwell Publishing, Oxford. In press.

Wellmer, F. and Riechmann, J.L. (2005) Gene network analysis in plant development by genomic technologies. *Int. J. Dev. Biol.* **49**:745-759.

Wellmer, F., Alves-Ferreira, M., Dubois, A., Riechmann, J.L. and Meyerowitz, E.M. (2006) Genome-wide analysis of gene expression during early *Arabidopsis* flower development. *PLoS Genetics*. In press.

Publications acknowledging the laboratory

Covert, M.W., Leung, T.H., Gaston, J.E. and Baltimore, D. (2005) Achieving stability of lipopolysaccharide-induced NF-kappaB activation. *Science* **309**:1854-1857.

Gama, C.I., Tully, S.E., Sotogaku, N., Rawat, M., Clark, P.M., Vaidehi, N., Goddard, W.A., III, Nishi, A. and Hsieh-Wilson, L.C. (2006) Sulfation patterns of glycosaminoglycans encode molecular recognition and activity. *Nature Chem. Biol.* In press.

Taganov, K., Boldin, M.P., Chang, K-J. and Baltimore, D. NF-κB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. Submitted.

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Vikalo, H., Hassibi, B. and Hassibi, A. (2006) A statistical model for microarrays, optimal estimation algorithms, and limits of performance. IEEE Transactions on Signal Processing. Special Issue on Genomics Signal Processing) **54**(6):2444-2455.

Monoclonal Antibody Facility
Supervisor: Paul H. Patterson
Director: Susan Ker-hwa Ou
Staff: Shi-Ying Kou

The Monoclonal Antibody Facility provides assistance to researchers wishing to generate monoclonal antibodies (mAbs), ascites fluid or other tissue culture services. We also produce polyclonal ascites Abs by immunizing mice with antigen until the serum titer is high enough, then inducing the mice with sarcoma cells to obtain high titer polyclonal ascites fluid. This method can provide 10-18 ml polyclonal ascites fluid per mouse by using small amount of antigen. In addition to these service functions, the Facility also conducts research on the development of novel immunological techniques.

In its service capacity, the Facility produced Mabs for the following groups during the past year. The Hill lab (UCLA) obtained mAbs against full-length recombinant trypanin, which is essential for cell mobility of African Trypanosome. The Zinn lab obtained mAbs against PTP4EAP (extracellular domain of PTP4E fused to human alkaline phosphatase). The Simpson lab (UCLA) obtained mAbs against a recombinant protein involved in RNA editing from Leishmania mitochondria. Caroline Enns (Oregon Health & Science University) obtained mAbs against extracellular domain of a human protein involved in iron regulation. The Reiter lab (UCLA) obtained mAbs against an N-Cadherin GST fusion protein. The Reiter lab (UCLA) also obtained mAbs against the 4th extracellular domain of N-Cadherin. The Hsieh-Wilson lab obtained mAbs against three different chondroitin sulfate glycosaminoglycans (CS-E, CS-C, & CS-A), which are synthetic carbohydates, and the mAbs will be used to map them in human brain.

We are currently working with the following groups: the Strauss lab is trying to obtain polyclonal ascites fluid against NS-5 from West Nile virus. Eugene Zhukovsky (Xencor) is trying to obtain mAbs against human BAFF, a type II membrane protein which functions predominantly as a B cell survival factor and specifically promotes the proliferation and survival of activated B cells by inducing expression of pro-survival oncogenes. Hill (UCLA) is trying to obtain mAbs against Gas-11, which represents the mammalian counterpart of Trypanin. It is produced by various species of trypanosomes.

Publications citing the Facility

Christiansen, J.J., Rajasekaran, S.A., Inge, L., Cheng, L.R., Anilkumar, G., Bander, N.H. and Rajasekaran, A.K. (2005) *Molec. Cancer Therap*. **4**(5)704-714.

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Rees, D.C., Lee, A.T., Locher, K.P. and Pinkett, H.W. (2006) *FASEB J.* **20**:A1338-A1338.

Sharma, S., Falick, A. and Black, D. (2005) *Mol. Cell* **9**(4)485-496.

Tully, S.E., Rawat, M. and Hsieh-Wilson, L.C. (2006) *J. Amer. Chem. Soc.* In press.

Nucleic Acid and Protein Sequence Analysis Computing Facility
Supervisor: Stephen L. Mayo
Staff: David R. Mathog

The Sequence Analysis Facility (SAF) provides software, computers, and support for the analysis of nucleic acid and protein sequences. Currently the SAF hardware consists of a Sun Netra running Solaris, a small 20 node Beowulf cluster, a file server, a 26 ppm duplexing laser printer, and a 16 ppm duplexing color laser printer. The PCs that comprise the "structure analysis facility" are also located in our facility. The SAF has over 250 registered users distributed among 50 research groups.

Most common programs for sequence analysis are available on the primary server http://saf.bio.caltech.edu/. These include the GCG and EMBOSS Packages, PRIMER3, Phred, Phrap, Cross Match, Phylip, and HMMER. Many of these may be accessed through the W2H or Pise web interfaces. Other programs, custom written programs, or special databases are available on request. The PCs support hardware stereo under both Linux and Windows XP. Under Linux the programs O, Molscript, XtalView, CCP4, and Delphi are available. Under Windows XP Swiss PDB Viewer, O, PyMol, POVray, and various drawing and animation programs may be used. The searchable documentation for these programs is available on the SAF web server. The lecture notes and homework from the introductory course "Fundamentals of Sequence Analysis" are also available on the SAF web server. BLAST jobs submitted through the SAF web interface run on the SAF Beowulf cluster (in parallel) faster than they do at the NCBI server. Personal sequence databases of up to 50 Mb may now be searched locally. An enhanced parallel HHMER server offers the full set of HMMER programs plus the unique ability to search any of the installed BLAST databases with an HMM.

Traces and sequences from the DNA sequencing facility may be analyzed on or downloaded from our server. The SAF also distributes site-licensed software for PCs and Macs like DNASTAR, Gene Construction Kit, ChemSketch, and X-Win.

Protein Expression Center
(http://www.its.caltech.edu/~pec)
Director: Jost Vielmetter
Staff: Inderjit Nangiana
Staff: Chris Fogelsong
Supervisors: Pamela J. Bjorkman, Barbara Wold

The Protein Expression Center was established in 1996 to meet the needs of the Caltech community for protein expression and purification. The services included expression of recombinant proteins in a variety of expression systems and purification of the expressed proteins. To date, the majority of recombinant proteins that have been produced at the Center have been expressed in the insect cell/baculovirus system, which is the most widely used and versatile eukaryotic system for the generation of recombinant proteins. This system has a number of benefits over other expression systems including the preservation of many glycosylation and other post-translational modifications that are important for protein function. Since its inception, the Center has generated over 900 recombinant viruses and has generated an average of 300 liters of infected viral cells per year.

More recently the need to use additional expression systems has brought mammalian expression systems into focus. To overcome limitations of the baculovirus system, particularly in cases where proper glycosylation of the Fc-portions of antibodies is required, transient expression in HEK-293 cells is an excellent alternative. We are currently validating two cell lines HEK293T and HEK293EBNA mostly for engineered antibody expression in the context of the "Engineering Immunity" program and have successfully expressed an anti-HIV antibody. Both cell lines have also been used successfully for transient expression of many different mammalian proteins.

Protein Expression, Purification, and Applications Involving Recombinant Proteins

A number of the recombinant proteins that have been expressed have also been purified by the facility staff over last years. The purification methodology has ranged from affinity chromatography to ion exchange or size exclusion chromatography, or a combination of these. The proteins that have been expressed and purified are diverse in their biochemical and functional characteristics, ranging from nuclear proteins involved in DNA replication to cell surface proteins mediating cell-cell interactions. Proteins expressed and/or purified by the Protein Expression Center have been used for a variety of experiments or applications. Some have been used as immunogens for the generation of antibodies, or to study or utilize their functional activity. In other projects, the Center has generated up to 30 different variants of a protein in order to allow investigators to do an exhaustive site-directed mutagenesis study to map a binding site. Many other proteins have been used in crystallization trials to solve their three-dimensional structures, resulting in the determination of several crystal structures by the Bjorkman laboratory.

Utilization of facilities: The utilization of the various services the Protein Expression Center has provided during the past year are summarized in the following list:

Service	Volume/orders completed
Baculovirus generation	135
Generation of high titer baculovirus stock	34 Liters
Baculovirus titer determination	23
Baculovirus plaque purification	4
Baculovirus protein expression and protein characterization	255 Liters
Protein purification	17

During that period the Center has provided services for the following Caltech investigators: David J. Anderson, Pamela J. Bjorkman, Judith L. Campbell, Raymond J. Deshaies, Linda Hsieh-Wilson, Mary B. Kennedy, Stephen L. Mayo, Carl Parker, Shan Shu-Ou, James H. Strauss, Paul W. Sternberg, David Tirrell, Alexander J. Varshavsky, and Kai Zinn.

Outside Users	Institution
John Arnez	University of Louisville
Betty Chen	UCLA
Yuan-Hung Chien	University of Illinois at Urbana
Sarah Conner	MIT
D. Eisenberg	Institute of Genomics and Proteomics, L.A.
Carol Flowers	University of Kansas Medical Center
Andy Herr	University of Cincinnati College of Medicine
Chih-Pin Liu	City of Hope
Minni McMillan	USC
Shlomo Melmed	Cedars-Sinai Medical Center, UCLA
Gopal Periyannan	Medical College of Wisconsin
Homa Sadeghi	BioRexis (VPR &D) PA
Mark Worwood	U.K.
Larry Zipursky	UCLA

Publications

Philipps, B., Forstner, M. and Mayr, L.M. (2005) A baculovirus expression vector system for simultaneous protein expression in insect and mammalian cells. *Biotechnol. Prog.* **21**:708-711.

Philipps, B., Rotmann, D., Wicki, M., Mayr, L.M. and Forstner, M. (2005) Time reduction and process optimization of the baculovirus expression system for more efficient recombinant protein production in insect cells. *Prot. Expr. Purif.* **42**:211-218.

Publications acknowledging the Expression Center

Davis, M.I., Bennett, M.J., Thomas, L.M. and Bjorkman, P.J. (2005) Crystal structure of prostate-specific membrane antigen, a tumor marker and glutamate carboxypeptidase. *Proc. Natl. Acad. Sci. USA,* **102**:5981-5986.

Hamburger, A.E., West, Jr., A.P., Hamburger, Z.A., Hamburger, P. and Bjorkman, P.J. (2005) Crystal structure of a secreted insect ferritin reveals a symmetrical arrangement of heavy and light chains. *J. Mol. Biol*. **349**:558-569.

Protein Micro Analyical Laboratory
Director: Jie Zhou
Associate Biologist: Felicia Rusnak
Faculty Advisor: Kai Zinn

Activities
Mass spectrometry of large biomolecules and small organic molecules
Proteomics (In-gel chemical and enzymatic protein digestion; LC/MS/MS and data base search)
Protein (Edman) chemical sequencing
De novo peptide sequencing by mass spectrometry
High performance liquid chromatography (HPLC)

Equipment
Quadrupole time-of-flight mass spectrometer (ABI QstarXL)
Triple quadrupole mass spectrometer (MDS Sciex API 365)
MALDI-TOF mass spectrometer (ABI Voyager de.str)
Capillary protein sequencer (Procise cLC, ABI 492)
HPLC nanoflow, 2D (Eksigent)
HPLC (ABI microbore 140D pump, PE UV monitor)
Laser puller (Sutter Labs)
MASCOT server

New Applications
For one project of Dr. Tirrell's group, we used LC Q-Tof MS to perform targeted detection of peptides with unnatural amino acid residues from digestion mixture of proteins with over 300 peptides. High mass accuracy and pre-selected masses in acquisition method enable us to selectively pick up expected peptides for MS/MS fragmentation during LC/MS run. Similar approach was also applied to detect peptides with two different alkylations on cysteine residues in the peptide mixtures for Varshavsky's group.

Some protein identification work was done for samples in gel pieces from groups of Simon and Parker, with just near the threshold of our instrument sensitivity to identify those proteins. Increased sensitivity by adding heated chamber on EIS ion source of Q-Tof would improve the work.

API 365 quadrupole ESI mass spectrometer has higher interface pressure (more collisions) than Q-Tof. Cooler interface of Q-Tof tends to produce more multiply-charged ion peaks of large proteins. So higher mass accuracy, higher sensitivity and more multiply-charged ion peaks for deconvelotion of ESI Q-Tof MS make it better instrument for mass spec analyses of large proteins. This application greatly improved the protein analyses for groups of Mayo, Gray and Winkler, with mass accuracy of +/- 1 Dalton for proteins of MW up to 20 kDa.

Application of negative-ion electrospray ionization in non-aqueous solvent enables us to analyze sulfated carbohydrates from Dr. Hsieh's group. Metal complexes from Barton's group were also analyzed in positive ion mode.

Our homemade, easily replaceable set-up for sample infusion and spray tip makes our ESI source feasible to analyze crude reaction products of organic compounds from groups of Barton, Dougherty, Grubbs, Dervan, Hsieh, Fraser, Tirrell and Peters.

Throughput and Interactions
During the first seven months of fiscal 2005 PPMAL interacted with 25 laboratories. Samples were analyzed from the Division of Biology, and Chemistry and Chemical Engineering (see list). A total of 1634 samples were analyzed. In addition to our work for campus faculty and staff, work was also performed for Nanogen, Geron, UCLA and Xencor (46 samples analyzed).

Mass Spectrometry
In seven months, 1,531 proteins, peptides, oligonucleotides, and carbohydrates, small organic compounds have been analyzed. This extrapolates to an annual throughput of over 2,600. Our off-campus activity recorded 26 samples.

Proteomics
For the period covering this report, 85 digests had been analyzed. This amount is over twice of last year's during the same period. This extrapolates to an annual throughput of 130 samples.

Protein and Peptide Sequence Analysis
The lab has sequenced proteins and peptides for 146 cycles, a 37% decrease over the same period last year. The average number of residues per sample rose from six to eight.

List of Facility Users and Activity

October 2005 - April 2006
On-Campus

Name	# Samples	# Mass	# Protein ID	# Sequences	# Cycles	# HPLC
Barton	534	525	9			
Bjorkman	1			1	6	
D. Chan	2	1		1	6	
Collier	12	12				
Davis	9	9				
Dervan	67	67				
Dougherty	104	104				
Fraser	19	19				
Gray	114	113	1			
Grubbs	8	8				
Heath	13	12		1	3	
Hsieh-Wilson	408	408				
Lester	5	5				
Mayo	159	158	1			
Newman	15		15			
Parker	1		15			
Peters	19	19				
Rees	21		27	1	10	
Roberts	8	8				
Schuman	2	2				
Simon	17		11	5	52	1
Tirrell	50	46		4	12	
Varshavsky	7		6	1	5	
Winkler	18	14		4	42	
Zewail	1	1				
TOTALS	**1634**	**1531**	**85**	**18**	**136**	**1**

Off-Campus

Geron	4	4				
Nanogen	10	10				
UCLA	5	1	1	3	50	
Xencor	27	11		14	96	2
TOTALS	**46**	**26**	**1**	**17**	**146**	**2**

Graduates

DIVISION OF BIOLOGY
DOCTOR OF PHILOSOPHY - 2006

Jordan Benjamin, Ph.D.
Biology
B.S., University of California, Santa Cruz, 2001
Thesis: Structural Studies of Human Immunodeficiency Virus Type I by Cryro-Electron Tomography

Baris Bingol, Ph.D.
Biology
B.S., Bilkent University, 2000
Thesis: Ubiquitin Proteasome System at the Synapse

Bede Michael Broome, Ph.D.
Biology
B.A., Princeton University, 1999
Thesis: Population Coding and Reconstruction of Complex Stimuli in the Locust Olfactory System

Ronald McKell Carter, Ph.D.
Biology
B.S., The University of Utah, 1998
Thesis: Explicit and Implicit Processes in Human Aversive Conditioning

Eun Jung Choi, Ph.D.
Biology
B.S., Seoul National University, 1998; M.S., 2000
Thesis: Development and Applications of Computational Protein Design

Gregory A. Cope, Ph.D.
Biochemistry and Molecular Biophysics
B.S., University of California, Santa Cruz, 1998
Thesis: Regulation of SCF Ubiquitin Ligases by Jab1/Csn5 and the Cop9 Signalosome

Johannes Graumann, Ph.D.
Biology
Diplom, Universität Konstanz, 2000
Thesis: Implementation of Multidimensional Protein Identification Technology and Its Application to the Characterization of Protein Complexes in Bakers Yeast

Harry Miguel Green, Ph.D.
Biology
B.S., University of California, Riverside, 1999
Thesis: Novel Methods for Studying Ras/Erk MAP Kinase Signaling in Developing T Cells

Erick Griffin, Ph.D.
Biology
B.A., Swarthmore College, 1996
Thesis: Mechanisms of Mitochondrial Fusion and Fission

Houman David Hemmati, Ph.D.
Biology
B.S., Stanford University, 1996
Thesis: Neural Stem and Progenitor Cells in Cancer and Development

Rajan P. Kulkarni, Ph.D.
Biochemistry and Molecular Biophysics and History and Philosophy of Science
B.A., B.S., Stanford University, 2000
Thesis: Mechanics of the Cytoskeleton: Examining the Dynamics of Cytoplasmic Transport through Fluorescence Microscopy

Christopher J. Lacenere, Ph.D.
Genetics
B.S., Carnegie Mellon University, 1994; M.S., California Institute of Technology, 2000
Thesis: Advances in Single Molecule Nucleic Acid Sequencing

Brian Lee, Ph.D.
Biology
B.S., University of Southern California, 1999
Thesis: Neural Computation of Self-Motion from Optic Flow in Primate Visual Cortex

Carolina Becker Livi, Ph.D.
Developmental Biology
B.Sc., Universidade Federal do Rio Grande do Sul, 1996; M.S., California Institute of Technolgy, 2000
Thesis: Spblimp1/krox: A Transcriptional Regulator with a Central Role in Endomesoderm Specification in Sea Urchin Embryos

Jessica Mao, Ph.D.
Biochemistry and Molecular Biophysics
B.S., State University of New York at Stony Brook 2000; M.S., California Institute of Technology, 2006
Thesis: Applications of Computational Protein Design

Joshua Scott Marcus, Ph.D.
Biochemistry and Molecular Biophysics
B.S., University of Florida, 2001; M.S. California Institute of Technology, 2006
Thesis: Single Mammalian Cell Gene Expression Analysis Using Microfluidics

Patricia A. Neil, Ph.D.
Computational and Neural Systems
B.E., University of Colorado, 1998
Thesis: Development of Audiovisual Integrations in Human Infants: The Effects of Spatial and Temporal Congruency and Incongruency on Response Latencies

Eric Michael Slimko, Ph.D.
Computational and Neural Systems
B.S.E., The University of Michigan, 1994
Thesis: Selective Silencing of Vertebrate Neurons: Strategies Using Invertebrate Ligand–Gated Ion Channels

Karli Kiiko Watson, Ph.D.
Biology
B.A., Swarthmore College, 1997
Thesis: The Von Economo Neurons: From Cells to Behavior

Daw-An Wu, Ph.D.
Biology
B.A., University of California, Berkeley, 1998
Thesis: How Perception Adheres Color to Objects and Surfaces: Studies Using Visual Illusions and Transcranial Magnetic Stimulation

Eric Stafford Zollars, Ph.D.
Biochemistry and Molecular Biophyics
B.S., University of Maryland, 1999
Thesis: Force Field Development in Protein Design

MASTER OF SCIENCE, BIOLOGY - 2006

Daniel R. Cleary, *Biology*

BACHELOR OF SCIENCE, BIOLOGY - 2006

Rebecca Abigail Adler*, *Biology and History and Philosophy of Science*
Chika Arakawa*, *Biology*
Timothy Robert Barnes, *Biology*
Michael Kae-Uei Chang, *Biology and Business Economics and Management*
Grace Chuang*, *Biology*
Angelina Marie Crans*, *Biology*
Mithun Diwakar*, *Biology*
Elena Fabrikant*, *Biology and Mathematics*
Meng-meng Fu*, *Biology*
Katherine Gabriela Gora*, *Biology*
Theresa Marie Grieco*, *Biology*
Maria Kwan Ling Ho, *Biology*
Po-Yin Samuel Huang*, *Biology*
Qinzi Ji*, *Biology*
David Alexander Kahn, *Biology*
Felicia Rachel Katz*, *Biology*
Dorota Zuzanna Korta*, *Biology*
Daniel Jacob Koslover*, *Biology*
Esther Sunghee Lee*, *Biology and English*
Jeremy Michael Leibs*, *Engineering and Applied Science (CNS) and Control and Dymanical Systems (Minor)*
Jun Lu*, *Economics and Biology*
Smaranda Constanta Marinescu, *Chemistry and Biology*
Shelby Anne Montague*, *Biology*
Colleen Lindsay Moody, *Biology*
Eva Rose Murdock, *Biology*
Paul Najime Nagami*, *Biology*
Kimberly Julia Popendorf, *Geobiology*
Ransom Harold Poythress, *Biology*
Adam Daniel Reeves*, *Biology*
Naboon Riddhiraksa, *Biology*
Tharathorn (Joy) Rimchala*, *Biology and Engineering and Applied Science*
Meru Jyoti Sadhu, *Biology*
Jessica Jan Stockburger, *Biology*
Emma Lynn Thomas*, *Biology*
Bingni Wen*, Biology
Regina L. Wilpiszeski, *Geobiology*
Wensi Xu*, *Biology and Business Economics and Management*
Mi Mickey Yang, *Biology*
Zhu Irene Ying, *Biology*
Shuhao Zhang*, *Biology*
Corinna Clio Markenscoff Zygourakis*, *Biology and English*

*Students whose names are followed by an asterisk are being graduated with honor in accordance with a vote of the faculty.

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ERATO
European Molecular Biology Organization
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Horowitz Foundation
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Human Brain Project
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Human Frontiers Science Program
Human Frontiers Science Program Organization (HFSP)
Huntington Hospital Research Institute
Huntington's Disease Society of America

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Instituto Gulbenkian de Ciência

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Jane Coffin Childs Memorial Fund for Medical Research
Japan Science and Technology Agency
Japanese Society for the Promotion of Science (JSPS)
Carl Zeiss Jena
Jet Propulsion Laboratory
Johnson & Johnson
Josephine V. Dumke Fund
Joyce Charitable Fund
Joyce Fund for Alzheimer's Disease Research
Juvenile Diabetes Research Foundation

Keck Discovery Fund
W.M. Keck Foundation
W.M. Keck Foundation for Discovery in Basic Medical Research
Kenneth T. and Eileen L. Norris Foundation
Kroc Foundation

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Leonard B. Edelman Discovery Fund
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Luce Foundation

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Margaret E. Early Medical Research Trust
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McKnight Foundation
McKnight Neuroscience of Brain Disorders Award
Merck & Co., Inc.
Mettler Fund for Autism
The Millard and Muriel Jacobs Family Foundation
Mind Science Foundation
The Betty and Gordon Moore Foundation
Moore Discovery Grant Program
Moore Foundation
Muscular Dystrophy Association

NASA
NASA/AMES
National Aeronautics and Space Administration
National Alliance for Autism Research
National Alliance for Research on Schizophrenia and Depression
National Association for Research in Schizophrenia and Depression
National Cancer Institute
National Eye Institute
National Geospatial Intelligence Agency
National Heart, Lung and Blood Institute
National Human Genome Research Institute
National Institute for Biomedical Imaging and Bioengineering
National Institute for Neurological Diseases and Stroke
National Institute of Allergy and Infectious Diseases
National Institute of Arthritis and Musculoskeletal and Skin Disease
National Institute of Child Health and Human Development
National Institute of General Medical Science
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National Institute of Mental Health, USPHS
National Institute of Neurological Disorders and Stroke
National Institute on Aging
National Institute on Deafness and Other Communication Disorders

National Institute on Drug Abuse
National Institutes of Health, (NINDS, DE)
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Alfred P. Sloan Foundation
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Stanley Medical Research Institute
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Substance Abuses and Mental Health Services Administration
Swartz Foundation
Swartz-Sloan Foundation
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TRDRP

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University of Washington/ONR

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Yuen Grubstake Fund

Ernest D. Zanetti Fund
Carl Zeiss Microimaging

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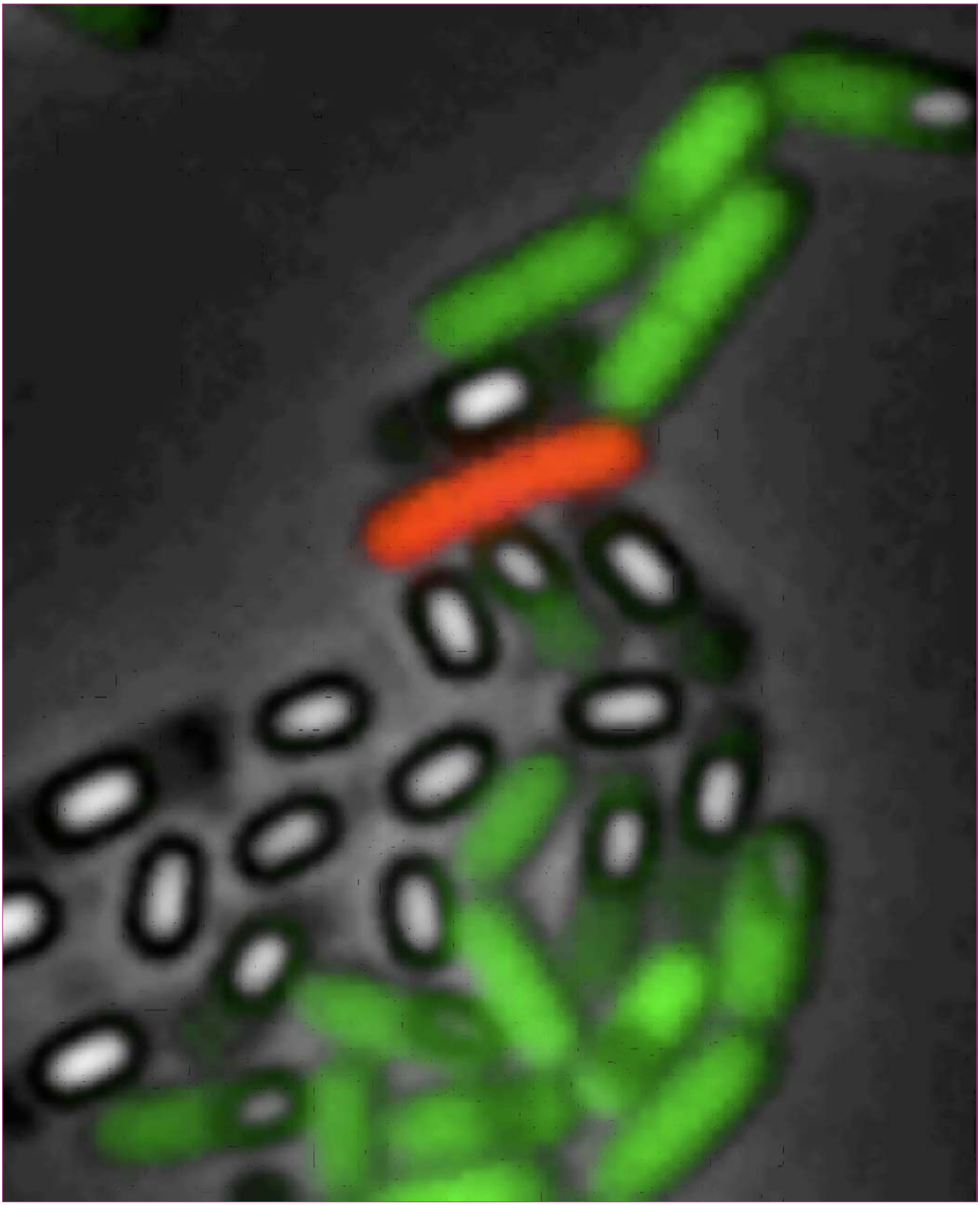

mage depicts a small clonal micro-colony of *Bacillus subtilis* cells. In these conditions, at least three different cell types are visible simultaneously: some cells (green fluorescence) are growing vegetatively while others (red fluorescence) are genetically competent (able to take up DNA from the environment). Still others have initiated a developmental program of sporulation (white objects). Recent results have shed light on the genetic circuitry that allows individual cells to decide randomly among these fates. (Michael Elowitz, Assistant Professor of Biology and Applied Physics)