OIST Annual Report 2008

Okinawa Institute of Science and Technology
Promotion Corporation

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Annual Report 2008

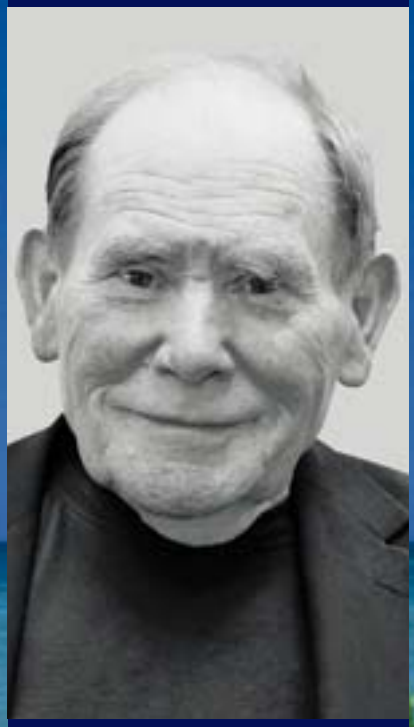
Preface

A major activity this year was the preparation of the legislation for the new OIST University. To establish the background for this, the OIST University Preparation Working Group prepared a Proposed Structure of the Graduate University, built on the original OIST Framework document, which offered possible academic models for the graduate program. The Working Group also surveyed several other leading graduate programs in science and technology in Japan and around the world. The BOG then summarized key elements of the proposed program in the Blueprint of the New Graduate University, which was handed to then Minister of State for Okinawa Affairs Fumio Kishida in July 2008. The Cabinet Office prepared the legislation, titled "Okinawa Institute of Science and Technology School Corporation Act", which was approved by the Cabinet for submission to the Diet in March. The Act is now under deliberation, and we look forward to its passage. During this period, many politicians and other government members came to visit, and the staff and researchers at OIST did an outstanding job of presenting our progress and plans for the future.

We added two new Principal Investigators this year and introduced Independent New Investigator appointments for young scientists, which are central to our commitment to enable the best young scientists from Japan and around the world to initiate their own independent research projects. Work is proceeding rapidly on the new campus in Onna-son. The first buildings and laboratories will be ready for occupancy in about January 2010. The design is striking and blends well with the beautiful natural environment of the campus. The interiors have been designed to encourage multidisciplinary research and interactions among the scientists. There will be excellent classroom and conferencing facilities.

As part of our introduction of environmental sciences as an area of focus at OIST, we co-sponsored a Satellite Workshop on Environment and Energy Issues in conjunction with the G8 Science and Technology Ministerial held in Okinawa, with participation from several members of the BOG. This year, we also greatly expanded our capacity in genomic analysis and are now undertaking projects on diversity and evolution in marine organisms.

We enthusiastically continue our progress toward opening the new university, which will play an important role in the future of Okinawa.



Sydney Brenner

President
Okinawa Institute of
Science and Technology
Promotion Corporation

During fiscal year 2008, two additional research units were established. The new research units are:

1. Marine Genomics Unit
   Principal Investigator: Dr. Noriyuki Satoh
   Establishment date: April 2008

2. Physics and Biology Unit
   Principal Investigator: Dr. Jonathan Miller
   Establishment date: April 2008

General Report

I. OIST P.C. Events

The Okinawa Institute of Science and Technology Promotion Corporation (OIST P.C.) was established on September 1st of 2005. The Corporation was established in order to conduct outstanding research and to prepare for the opening of a world-class graduate university of science and technology in Okinawa.

The main events in FY2008 were as follows:

June 14, 2008	The G8 Science and Technology Ministerial Satellite Workshop on Environment and Energy Issues was held in Okinawa.
July 28-30, 2008	The 6th Board of Governors meeting was held in Okinawa and Tokyo. Recommendations for the overall Blueprint for establishing the new graduate university were submitted formally to then Minister for Okinawa Affairs Mr. Fumio Kishida.
November 9, 2008	The Open House 2008 for the Okinawa community was held.
February 16-17, 2009	The 7th Board of Governors meeting was held in Tokyo.
March 3, 2009	The Okinawa Institute of Science and Technology School Corporation Act was approved by the administration of Prime Minister Taro Aso in a Cabinet meeting and submitted to the Japanese Diet.

II. BOG Meeting Summary

The Board of Governors (BOG) was established under the Corporation Act in 2005. The tasks of BOG are to oversee the Corporation and advise the President. BOG operates within Articles 12 and 13 of the Corporation Act. As of March 31st, 2008, there are 10 Board members, with Dr. Arima and Dr. Wiesel serving as co-chairs of the Board since December 2006. Dr. Jean-Marie Lehn resigned in August 2008 and Dr. Steven Chu resigned in February 2009 after he was appointed as Secretary of the U.S. Department of Energy in the administration of President Barack Obama in Washington, D.C..

<Members of the Board of Governors>

Dr. Akito Arima*	Chairman, Japan Science Foundation Former President, The University of Tokyo
Dr. Jerome Friedman	Professor, Massachusetts Institute of Technology Nobel Laureate (Physics, 1990)
Dr. Timothy Hunt	Cancer Research UK Nobel Laureate (Physiology or Medicine, 2001)
Dr. Ichiro Kanazawa	President, Science Council of Japan
Dr. Kiyoshi Kurokawa	Professor, National Graduate Institute for Policy Studies Former Special Cabinet Adviser Former President, Science Council of Japan
Dr. Yuan-Tseh Lee	President Emeritus, Academia Sinica Nobel Laureate (Chemistry, 1986)
Sir Martin Rees	President, The Royal Society
Dr. Hiroko Sho	Professor Emeritus, The University of the Ryukyus
Dr. Susumu Tonegawa	Professor, Massachusetts Institute of Technology Nobel Laureate (Physiology or Medicine, 1987)
Dr. Torsten Wiesel*	President Emeritus, Rockefeller University Secretary General, Human Frontier Science Program Organization Nobel Laureate (Physiology or Medicine, 1981)

* Co-chairs

The 6th BOG Meeting

The 6th BOG meeting was held on July 28th and 29th, 2008 in Okinawa and on July 30th in Tokyo.

On July 28th, the BOG members received a background presentation on the campus construction and later participated in the campus construction site tour. In the afternoon, the members received a presentation of their research from several OIST P.C. Principal Investigators (PIs).

Attending Board members of the main session on July 29th were: Dr. Akito Arima, Dr. Jerome Friedman, Dr. Timothy Hunt, Dr. Ichiro Kanazawa, Dr. Kiyoshi Kurokawa, Dr. Yuan-Tseh Lee, Dr. Hiroko Sho, Dr. Susumu Tonegawa, and Dr. Torsten Wiesel. Invited guests were Okinawa Governor Mr. Hirokazu Nakaima and Mr. Osamu Shimizu, Director-General of the Okinawa Development and Promotion Bureau, the Cabinet Office, as well as Mr. Gordon Hatton of BLL. Observing guest was Mr. Koji Omi, former Minister of State for Okinawa and Northern Territories Affairs, Science and Technology Policy.

The following items were on the agenda of the official session of the meeting on July 29th.

- Governor of Okinawa, Mr. Nakaima, gave a greeting.
- OIST P.C. President Dr. Brenner reported on progress of OIST P.C..
- The Co-chairs, Drs. Wiesel and Arima, reported on the outcome of the BOG working group meeting on university planning, held on May 12th, 2008.
- Mr. Shimizu of the Cabinet Office reported on the new medium-term objectives of OIST P.C. and FY2009 budget for OIST P.C..
- OIST P.C. Executive Director Dr. Baughman reported on activities of OIST P.C., such as survey of international graduate universities and the G8 satellite workshop on environment & energy issues, which took place on June 14th, 2008 in Okinawa.
- Mr. Hatton of BLL reported on the campus development including the main campus construction progress and the Seaside Faculty Housing.
- The executive session took place after the official session in the morning.

In the morning of July 30th, Drs. Wiesel and Arima formally submitted the Blueprint of the graduate university to then Minister for Okinawa Affairs Mr. Fumio Kishida, which had been finalized during the BOG meeting. In the afternoon, a press conference was held by the co-chairs to provide a summary of the 6th BOG meeting and explain to reporters about the Blueprint.

The BOG members submitted the Blueprint to then Okinawa Affairs Minister Kishida

The 7th BOG Meeting

The 7th BOG meeting was held on February 16th and 17th, 2009 in Tokyo. Attending Board members were: Dr. Akito Arima, Dr. Jerome Friedman, Dr. Ichiro Kanazawa, Dr. Kiyoshi Kurokawa, Dr. Yuan Tseh Lee, Dr. Hiroko Sho, Dr. Susumu Tonegawa, and Dr. Torsten Wiesel. Invited guests were Minister for Okinawa and Northern Territories Affairs Mr. Tsutomu Sato, Okinawa Governor Mr. Hirokazu Nakaima, Mr. Osamu Shimizu, Director-General of the Okinawa Development and Promotion Bureau, the Cabinet Office, and Mr. Gordon Hatton of BLL. Observing guest was Mr. Koji Omi, former State Minister for Okinawa and Northern Territories Affairs, Science and Technology Policy.

The following items were covered in the meeting agenda on February 17th.

- Minister Sato and Governor Nakaima made opening remarks.
- The Co-chairs, Drs. Wiesel and Arima, gave an update on the BOG membership.
- The Co-chairs, Drs. Wiesel and Arima, reported on the outcome of the BOG working group meeting in October 2008.
- Mr. Shimizu of the Cabinet Office reported on FY2009 budget for OIST P.C., the Okinawa Institute of Science and Technology School Corporation Act, and the new medium-term objectives of OIST P.C..
- OIST P.C. President Dr. Brenner and Executive Director Dr. Baughman reported on the new medium-term plan for OIST P.C. and the university planning.
- Mr. Hatton of BLL reported on the new campus construction.

At the end of the day, a press conference by the co-chairs took place to provide a summary of the 7th BOG meeting.

III. Campus Master Plan Status

Civil Works

The site development work for the second cluster, "Laboratory 2 and Laboratory 3", located on the hill opposite the first cluster site, was completed in July 2008 and five of the seven bridges connecting the various hills were completed by September 2008.

The infrastructure works such as electric power lines, water supply pipes, and sewage pipes, were commenced in FY2008.

The tunnel or gallery and a vertical elevator shaft are one of the most characteristic features of the campus plan. They are the principal connection between the "Village Zone" and the "Laboratory Zone". The construction of the tunnel and the vertical shaft was completed in July 2008.

The site development work for the hillside housing was also commenced in FY2008.

Environment Impact Assessment

Throughout the construction, the environmental impact was monitored carefully. This campus project was subject to an Environmental Impact Assessment (EIA) process. The contractors were required to follow the environmental-management instructions, which were established in the course of the EIA process. Additionally, a third party consultant was hired to monitor and investigate the environmental impact and also to instruct the contractors how to minimize impact on the campus site environment during their construction. The construction progressed in compliance with Okinawa Prefectural Ordinance for Prevention of Red Clay Outflow, and careful coordination and planning were required to prevent the discharge of turbid storm water runoff to the local rivers. This was achieved by implementing turbid water treatment facilities and inspection patrols on rainy days. In FY 2008, OIST P.C. submitted a report of environmental impact survey to Okinawa Prefecture and opened the report to the public.

Facility Construction

The structure and exterior for Laboratory 1 and Center Building were contracted in March 2008 and the mechanical, electrical and plumbing works were contracted in May 2008. These works made good progress during the fiscal year. The structure and exterior are scheduled to be completed in June 2009, and completion of interior work will follow, with the aim of starting the research work in the campus by the end of FY 2009, after starting the move from the Uruma site.

The construction of the energy center, which provides electricity and city water to the laboratory buildings, was commenced in October 2008.

At the seaside campus, construction of eight faculty housing units was completed, and some units came into service in FY 2008. These faculty housing units will be utilized as accommodation for invited lecturers at academic workshops in the Seaside House, or for temporary accommodation for new principal investigators.

IV. Administration and Finance

Research Support

(1) Scientific Committees

Three meetings of the Animal Experiment Committee, one meeting of the Genetic Recombination Experiment Committee and one meeting of the Biosafety Committee were held. The Human Subjects Research Review Committee was newly established.

<Meetings of the experimental committees held in FY2008>

Dates	Title	Main Topics
April 16, 2008	The 8th meeting of the Animal Experiment Committee	- Review of four applications for ethical approval for use of animals - Revision of the regulations
July 16, 2008	The 9th meeting of the Animal Experiment Committee	- Review of two applications for ethical approval for use of animals - Revision of the regulations
August 13, 2008	The 5th meeting of the Genetic Recombination Experiment Committee	- Review of three applications for genetic recombination experiment plan

Dates	Title	Main Topics
August 13, 2008	The 2nd meeting of the Biosafety Committee	- Review of two applications for the handling of pathogens and toxins
October 23, 2008	The 10th meeting of the Animal Experiment Committee	- Review of one application for ethical approval for use of animals - Review of one notification of animal experiment completion - Revision of the regulations
November 11, 2008	The 1st meeting of the Human Subjects Research Review Committee	- Appointment of the chairperson - Establishment of the regulations - Review of one application for approval of human subjects research

(2) Competitive Research Funds

Status of Awarded KAKENHI (Grants-in-aid for Scientific Research) is as follows :

- Number of Awarded Grants : 8
- Amount : 17,225 K yen

In order to facilitate applications from non-Japanese researchers, information regarding KAKENHI and grants provided by JSPS (Japan Society for the Promotion of Science) was provided to researchers in both Japanese and English. In addition, support was provided for individual inquiries from non-Japanese researchers in English and for preparing applications in Japanese.

(3) Patent Application

An intellectual property seminar was held in December 2008 to increase awareness about intellectual property and patent applications in OIST P.C. by explaining the importance of patent and intellectual property to both researchers and the administrative staff. The total number of patent applications submitted thus far is 8.

(4) Joint Research, Externally Sponsored Research and OIST P.C.-sponsored research

The number of joint research projects, externally sponsored research projects and OIST P.C.-sponsored research projects is as follows.

<The number of joint research, externally sponsored research and OIST P.C.-sponsored research in FY2008>

	Partner Organization		Total
	Domestic institution	Foreign institution	
Joint research	12	4	16
OIST P.C. -sponsored research	1	0	1
Externally sponsored research	1	0	1

(5) Academic Exchange

Based on the academic exchange agreements with the University of the Ryukyus and Nara Institute of Science and Technology, exchange in the fields of education and research was promoted.

(6) Preparation of Research Manuals

For the convenience of researchers, the research support manual and several research-related regulations were printed and bound.

DNA Sequencing Center

The OIST DNA Sequencing Center was established in FY 2008. We operate four high through-put sequencing systems. Several research units have been using the facility as part of their research projects, including the Brenner, Sato and Yanagida units.

(1) Systems

- Roche GS FLX Titanium system (also referred to as "454")
- Illumina GA-II system (also referred to as "Solexa")

(2) Applications

- The center supports sequencing applications, including but not limited to :
- Whole genome (*de novo*) sequencing
- Re-sequencing for SNPs detection
- ChIP-sequencing
- Gene Expression analysis
- Small RNA discovery/expression
- Transcriptome analysis

(3) Projects Using the Center Services

- Acorn Worm Genome Project (Sato unit) : Whole genome sequencing of the Acorn worm (*Ptychodera flava*) by Roche FLX (May 2008 -).
- Amphioxus Genome Project (Sato Unit): Whole genome sequencing of the Amphioxus (*Branchiostoma floridae*) by Roche FLX (May 2008 -).
- Lamprey Genome Project (Brenner Unit): Whole genome sequencing of the Japanese lamprey (*Lethenteron japonicum*) with Roche FLX (April 2009 - March 2010).
  Genomic Approach to the Bleaching of Corals (Sato Unit): Whole genome sequencing of the
- Okinawan corals (*Acropora digitifera, Ctenactis echinata*), Genomic comparison with several corals in the world (*ex. Acropora millepora*) using Roche FLX and Illumina GA-II (February 2009 -).
- Pombe Mutation Library Analysis Project (Yanagida Unit): SNPs detection for the mutation library of the yeast (*Schizosaccharomyces pombe*) with Illumina GA-II (April 2009 -).
  Investigation of the Primitive Chordate Genome (Sato unit): ChIP-seq for urochordate ascidian (*Ciona intestinalis*) and acorn worm (*Ptychodera flava*) with Illumina GA-II (April 2009 -)

Finance

The total budget for FY2008 was 19,566 million yen, including the 1st and the 2nd supplementary budget, which was approximately 125% increase from the previous Fiscal Year, 8,702 million yen. The sizable budget increase was mainly attributable to the budget for construction and infrastructure improvement under the Subsidy for Facilities.

<Budget comparison between FY2007 and FY2008> (Million Yen)

	FY2007	FY2008
Subsidy for Facilities	4,419	14,942
1) Land Development	725	80
2) Construction	2,864	10,905
3) Infrastructure Improvement	650	3,814
4) Land Acquisition	180	143
Subsidy for Equipment	-	170
Equipment	-	170
Subsidy for Operations	4,283	4,454
1) Research Expenses, Workshops etc.	3,558	3,753
2) General Administrative Expenses	725	701
Grand Total	8,702	19,566

Note : FY2008 Original Budget Total 10,740 million yen
1st Supplementary Budget 4,568 million yen
2nd Supplementary Budget 4,258 million yen

Human Resources

The total number of full-time employees was 194 as of April 1st, 2009. Of these, 144 were in research positions, and 47 were international.

Staff Training :

To improve administrative capabilities, staff are provided with appropriate training opportunities. We

provided 30 professional, educational courses to employees in FY2008. This included Japanese classes to meet the needs of an increasing number of international researchers and technicians.

Rules and Regulations

The review of the current rules and regulations including the document management has been conducted to secure appropriate and effective administrative operations. Also the preparation work for the re-organization of the present administrative organization has started to ensure the smooth transition to the opening of graduate university.

IT Infrastructure

Computational resources for scientific research have been expanded to meet the needs of a growing research staff with increasing computational requirements. A new general purpose computing cluster has been installed and made available to researchers, increasing our core computational ability by over 500%. Additionally, a large-memory computer has been installed with one terabyte of core memory to accommodate the large data sets required for computational genomics and other disciplines with similar needs. A small test cluster has been installed to explore the possibility of using unique processor architectures for computationally intensive scientific problems.

Server virtualization has been implemented to reduce the number of servers. Common services have been consolidated onto shared servers to reduce the costs associated with space, power, and cooling. This has also resulted in increased availability by allowing services to be migrated between servers.

The rapid growth of data for both research and administrative activities is being addressed by an expanded data storage infrastructure. New storage technologies and policies are being implemented to ensure data confidentiality, security, and availability across the enterprise.

Networking technologies have been upgraded to enhance collaborative capabilities between research units, and between administrative personnel located at geographically separate locations. This has resulted in reduced costs and increased performance. Experiences with these technologies will help guide the new campus planning to ensure world-class networking infrastructure.

Community Relations and Public Relations

OIST P.C. provided various opportunities for local citizens, including school children, teachers, and lay people, to learn about our research and other activities through lectures, school visits, film shows, etc.

OIST P.C. held its first "Open House" in November 2008, providing an opportunity for local citizens to see firsthand the OIST research activities. Approximately 630 people came to the event, which included lectures by PIs, scientific exhibitions, demonstrations, and lab tours.

OIST P.C. Open House 2008 (entrance)

OIST P.C. Open House 2008 (demonstration)

Throughout the whole the year, OIST P.C. received many visitors, including those from abroad as well as officials from the central and local governments.

Three issues of newsletters and an annual report were published and distributed to the central and local governments, academic and research institutions at home and abroad, as well as to visitors to OIST P.C., including participants in OIST P.C.-sponsored workshops and events. In addition, we have revised our corporate brochure to reflect our current and future activities.

Information about the progress on the campus construction, changes to the organizational structure, workshops & seminars, recruitment and tenders, etc. was updated on the OIST P.C. Website.

	Types of activities	Number of activities
1	Lectures by PIs	12
2	Lectures by OIST P.C. officials / staff	3
3	Open House	1
4	Visits to OIST P.C.	48

V. Graduate University Preparation

A working group meeting to discuss the structure of the new graduate university was held with several members of the Board of Governors and others in May 2008. The recommendations of the working group were presented in the "Blueprint of the New Graduate University" describing the proposed structure and governance of the Graduate University at the BOG meeting in July 2008. The Blueprint was submitted to then Minister Kishida. Based on the Blueprint, OIST P.C., including the Graduate University Preparation Group, advanced the implementation of plans for the opening of the graduate university.

As a follow up to the FY2007 survey about graduate universities, OIST P.C. conducted another survey in FY2008 targeting different institutions. The survey items and target universities and graduate programs are as follows;

Survey items ;

- Education and research fields
- Organization structure, regulations, bylaws, etc.
- Personnel system for faculty and researchers
- Finance, including revenue / expenditure; remuneration structure of presidents, other officers, professor, and researchers; and student support

Target universities and graduate programs ;

- Massachusetts Institute of Technology (MIT)
- California Institute of Technology (Caltech)
- Stanford University
- University of Cambridge
- University of Tokyo
- Tokyo Institute of Technology
- Keio University

The OIST P.C. Graduate University Preparation Group, an internal study group, drafted a report about the possible structure for the graduate university, the "Proposed Structure of the OIST Graduate University," including research and educational organization, curricula, administrative operations, academic advisory committees and financial plans. This report was distributed to advisors and interested parties, including the BOG meetings in July 2008 and February 2009. Based on feedback on the report, OIST P.C. has continued to develop the plan for operation and accreditation of the new graduate university.

Scientific Report

Brain Mechanisms for Behaviour Unit

Principal Investigator:
Gordon Arbuthnott

Research Theme:
Cortical Influences on Striatal Cells

Abstract

The basal ganglia are composed of a series of subcortical nuclei. The caudate nucleus and putamen (striatum) and subthalamic nucleus receive major afferent connections from the cerebral cortex, midbrain and thalamus. The internal segment of the globus pallidus and the substantia nigra, pars reticulata form the output nuclei that send back information, modified by the action of dopamine, to frontal cortical areas via thalamic nuclei.

These brain regions are the main focus of the unit since they play critical roles in the planning, learning, and execution of behaviors. Many regions of the cortex project heavily to the basal ganglia. Many cortical cells contribute to firing striatal cells so any two individual cells selected at random for recording are unlikely to be synaptically connected. Therefore, to investigate the electrophysiology of these important central synapses individually, we need to examine them in cultures where pairs of cells are much more likely to be connected together. This year has seen important developments towards this aim both in the collaborations with the Universities of Ottawa and Otago, as well as the research here in Okinawa. We now have the first few records of these synaptic connections in culture, have detailed anatomical studies of the composition of the cultures, and have developed methods to look at the patterning of activity in the cultured cells.

We have also completed a study of a likely source of the therapeutic benefit from stimulation of the subthalamic nucleus for patients with parkinsonism.

1 Staff

Brain Mechanisms for Behaviour Unit

Researchers: Dr. Marianela Garcia Munoz, Dr. Fiona Randall (from January 27, 2009),
Takuya Hikima Ph.D. (from April 2009)
Technical staff: Sha Pandian M.Sc. (from June to December, 2008)
Research Administrator / Secretary: Ms. Hiroko Chinone

2 Partner Organizations

University of Ottawa

Type of partnership: Joint research
Name of principal researcher: Professor W. A. Staines
(visiting the Unit June 2009, on the left in the picture)
Name of researchers: Professor A. Krantis, Professor Geoff Mealing, Dr. Sarah Schock,
Kheira Jolin-Dahel, M.Sc.
Research theme: Isolating genetically marked neurons for culture

University of Otago

Type of partnership: Joint research

Name of principal researcher: Dr. Beulah Leich
Name of researcher: Olga Shevtsova, M.Sc.
Research theme: Determining the location of L-type Calcium channels (CaV1.3) on striatal membranes.

University of Otago
Type of partnership: Ongoing research funded in New Zealand
Name of principal researcher: Professor Gordon Arbuthnott
Name of researchers: Dr. Brian Hyland and Dr. Cyril Dejean
Research theme: Cortical effects of deep brain stimulation in the subthalamic nucleus

3 Activities and Findings

3.1 OTTAWA UNIVERSITY JOINT RESEARCH PROJECT

In Canada we have completed a study of the development of striatal interneurons in cultures of dissociated cells from embryonic rats and mice. In 2007 we had already seen that the cholinergic interneurons were missing in the cultures from E18-19 rats and had found that they could be cultured from earlier embryos (E13-14). Since the establishment of the collaborative grant we have employed a post doctoral worker in Ottawa who has examined the timing of mouse early development (so that we now know that E14-15 and E12-13 in the mouse is the equivalent of the E18-19 and E13-14 rat, and we can now do the equivalent experiments with embryonic cells in Okinawa). Together we have systematically studied the development of all the known types of interneurons in cultures of striatum, alone and in cultures with cortical neurones included. This study is being prepared for publication and is to be presented at the Experimental Biology Congress in New Orleans in April 2009. We now have the anatomical background for our developing electrophysiological study of the same cultures.

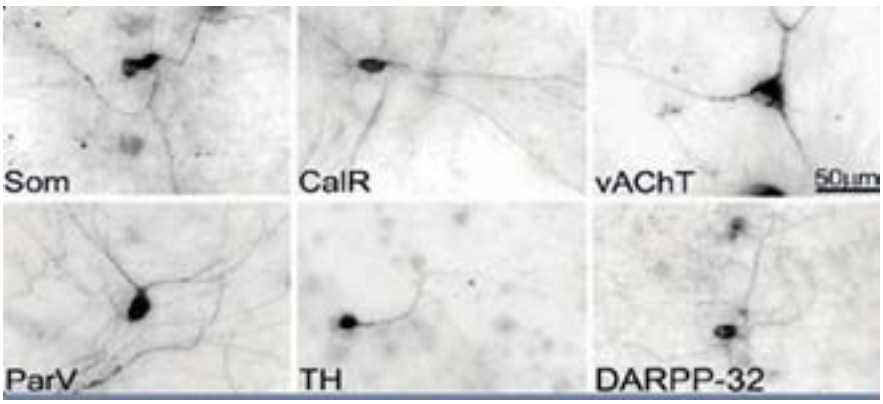


Figure 1: Each of the known cell types in the striatum are illustrated from cultures prepared from dissociated embryonic neurons, cryopreserved and then grown in culture for 5 weeks. Som - somatostatin interneurons; CalR- calretinin interneurons; vAChT- acetylcholinergic internurons; ParV- parvalbumin interneurons; TH-tyrosine hydroxylase containing interneurons; DARPP-32 (dopamine and adenylcyclase responsive phospho protein) marks the output neurons of the striatum

3.2 OTAGO UNIVERSITY JOINT RESEARCH PROJECTS

1-Neurological Foundation of New Zealand Project

The implications of our experiments, for understanding the mechanism of action of deep brain stimulation (DBS) as it is delivered to patients, are important since they suggest that the cortex and not the target (the subthalamic nucleus) is the site of action. We have tried - so far without convincing success - to produce recovery from dopamine receptor induced immobility in the rat by stimulation at other sites which should have a similar action on the cortex. The experiments are technically challenging, and did not produce a clear result. The stimulation in the pons, though it did help the animals recover somewhat from their catalepsy, caused movements by itself that both, limited the current we could use for stimulation, and confounded the clear interpretation of the recovery of mobility in the animals. Our preliminary report to the Society of Neuroscience 2007 has become a high profile paper in Cerebral Cortex published electronically this year and in the journal next year.
Dr. Cyril Dejean also explored the slow waves that accompany dopamine blockade in the EEG of awake rats. We have prepared a detailed account of the influence of dopamine antagonists on the

slow waves recorded from the globus pallidus and cortex and we will present it for publication in the new year, it was the subject of a poster presented to the Federation of European Neuroscience societies in Geneva this year.

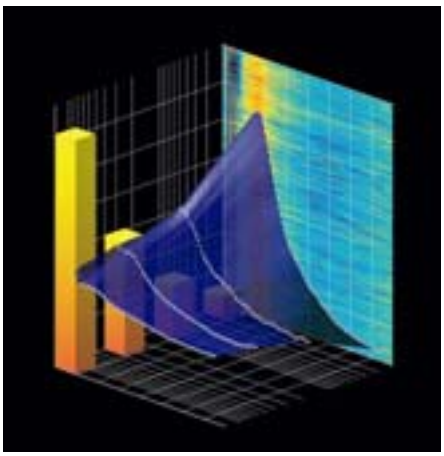


Figure 2: The transparent purple surface represents the shape of the increasing evoked potential as the stimulus increases. The yellow columns represent the time the rats stayed immobile on a bar 10 cm above the floor in spite of the stimulation. As the evoked potentials increase the time on the bar decreases. Statistical analysis suggests that the two are inversely correlated (R^2=0.84).

3.3 OTAGO UNIVERSITY JOINT RESEARCH PROJECTS

2- Ultrastructural joint research project

We are now supporting a student in Otago Department of Anatomy and Structural Biology to continue a project that is aimed at determining the ultrastructural localization of the calcium channel Cav1.3. This channel is present in the cells of the substantia nigra that make dopamine and also in the striatal cells that receive dopamine. It is implicated in the control of synapse number in the striatum and of the spontaneous activity in dopamine cells. Our preliminary findings suggest a synaptic location for the channel in striatum but we need many more images before we are confident about the quantitative aspects of this finding.

3.4 EXPERIMENTS IN OIST AT URUMA

We have developed in OIST both multielectrode array recordings and calcium imaging systems with which to study the patterns of electrical activity in the cultured cells and are beginning a detailed analysis of our results.

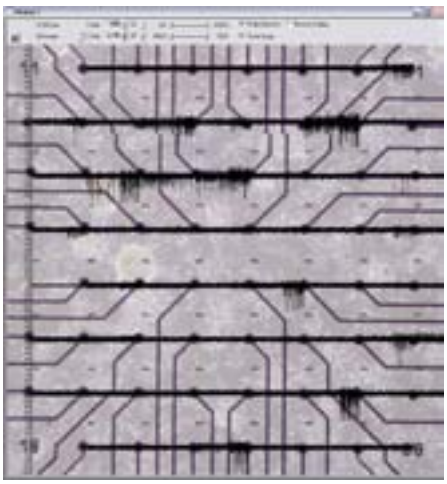


Figure 3: In this screen shot from a multielectrode recording experiment the cultured cells can be seen in the background. Mouse cortex and striatal cells E14-15 were plated over an array of 64 electrodes of 30μm diameter separated by 200μm. The electrical records of their activity are displayed as 1s long traces running to the right from the electrode to the next one on the grid. Neurons discharge as single spikes, bursts, multiunit activity or synchronous discharges.

3.5 Dynamical System Group

We are preparing a description of the spontaneous activity in corticostriatal cultures including previous studies in similar cultures done some years ago by myself with Dr Staines in Ottawa, and recent studies performed by us and also by Dr C. Vickers in OIST. Striatal cells in these cultures have a dramatic pattern of spontaneous activity characterised by bursts of activity very like those seen in vivo, but absent from slices. The striatal activity depends on the activation of AMPA type glutamate receptors and is modified by NMDA receptors. In order to have such activity in slices it is necessary to modify the medium and add NMDA to the perfusate. In culture, the activity is a normal part of their 'resting' behaviour and is also obvious on the multielectrode arrays (Figure 3).

We have also been studying the electrophysiology of single cells and pairs of corticostrial neurons in culture by patch clamp. We have evidence of inhibitory connections between striatal cells and excitatory connections between cortical and striatal cells (Figure 4).

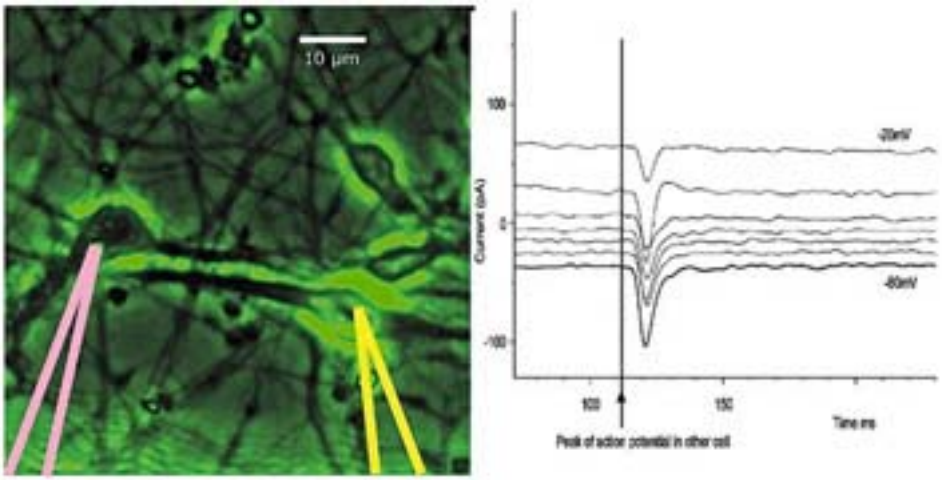


Figure4: The photomicrograph on the left shows cells from a culture with the fluorescence image superimposed so that cortical cells look green while the striatal cells in the culture are dark with a green halo because they are imaged in phase contrast. The electrode positions are drawn in for clarity; pink for the striatal electrode and yellow for the cortical one.

The traces on the right indicate the currents generated in a striatal neuron by single action potentials in a cortical neuron in a dissociated cell culture like the one illustrated. Each line is the average of 5 trials following an action potential in the cortical cell (at the vertical line in the figure) and the set includes records taken at holding potentials from -80mV in 10mV steps to -20mV. There is obvious spontaneous activity in the cultures which affects the averages, but it is clear that the responses do not reverse in direction over the set. These results suggest that the records are induced by an excitatory synaptic connection. Our first 6 pairs of connected cells of this type have an average inward synaptic current of 79.3±20.7pA at -80mV (thicker line in the figure).

During this financial year we have also upgraded our 2-photon microscope so that we will be able to use second harmonic imaging to study voltage changes in the cultured cells as well as the calcium imaging that we have already set up. I look forward to having time to develop that aspect of the work in the coming year. The single synapse project has occupied me for the past year but now it has been taken over very successfully by Dr. Fiona Randall who joined us in January this year.

3.6 Future plans

Next year should see us move into exciting new space on the Onna campus. Before then there will be both an expansion of our team and the beginning of three new projects in the Unit. Now that we have the cultured cells growing and the initial problems of their characterization solved, it is time to bring more personnel into the Unit to exploit the development of this more accessible model of corticostriatal pathways. Two new postdoctoral workers will join the research Unit and we will host a visit from an expert neuroanatomist in the next year:

1-Dr. Takuya Hikima.

In thinking about the control of synaptic strength in the striatum we have limited our investigations so far mainly to changes in the post-synaptic cells. Dr Hikima's appointment is intended to expand our knowledge of the actions on the other side of the synapse - on the presynaptic terminals. There is

ample evidence in the literature that transmitter release can be changed at corticostriatal synapses. Having completed his Ph.D. only this month Takuya is bringing to his first Post-doctoral position experience in the use of synaptopHlorins, molecular markers of pH which have given him information about the transmitter storage and release in the hippocampus. We hope to be able to use his expertise to examine the release of transmitters in the striatum both in slices and in cultures. In his latest publication Dr Hikima has also used newly developed channelopsins that will render cells sensitive to light. In collaboration with the Wickens' group, we have been developing a similar methodology. Dr. Hikima's access to, and experience with, newer versions of the original proteins used to transfect neurons in order to make them sensitive to light will make him an excellent person to bridge those projects and facilitate their use in a variety of relevant preparations.

2-Dr. Luis Carrillo-Reid.

Dr. Carrillo-Reid has recently published work describing the patterning of cellular activity in slices of the striatum. Having described the patterns in slices in which the activity had been increased by incubation in magnesium-free medium, he went on to describe the actions of acetylcholine on the development of patterns of striatal cell activity in the slices (Carrillo-Reid et al. 2008, 2009). We have the equipment in place to study the same phenomena in cultures where the activity is driven by much different cortical activity and with or without the presence of cholinergic cells. The conclusions that have been drawn about the drug induced firing pattern in slices, can then be compared with that seen in cultures in which the cholinergic influence is either absent or present.

3-Dr. Cali Ingham

Dr. Ingham is a long-time collaborator from my years in Edinburgh and is an expert electron microscopist with whom I have done many quantitative studies of the anatomy of the basal ganglia. She will take some three months research leave to work with us over the summer to establish methods and make vital ultra-structural observations in the corticostriatal cultures that we have developed in the Unit. We hope to be able to see individual corticostriatal connections in the cultures and compare them to the synapses formed in vivo. In particular we will look at the influence of dopamine on the structure of the corticostriatal synapses, since it has been suggested, from different kinds of cultures, that the normal development of spine synapses depends on the presence of dopamine neurones in the culture.

4 Publications

4.1 Journals

Dejean, C., Hyland, B., & Arbuthnott, G. Cortical Effects of Subthalamic Stimulation Correlate with Behavioral Recovery from Dopamine Antagonist Induced Akinesia. *Cerebral Cortex* (2009).

Shindou, T., Arbuthnott, G.W., & Wickens, J.R. Actions of Adenosine A2A Receptors on Synaptic Connections of Spiny Projection Neurons in the Neostriatal Inhibitory Network. *J Neurophysiol* 99 (4), 1884-1889 (2008).

Wright, A.K., Garcia-Munoz, M., & Arbuthnott, G.W. Slowly progressive dopamine cell loss; a model on which to test neuroprotective strategies for Parkinson's disease? *REVIEWS IN THE NEUROSCIENCES* (2009).

Wright, A.K., Miller, C., Williams, M., & Arbuthnott, G. Microglial activation is not prevented by tacrolimus but dopamine neuron damage is reduced in a rat model of Parkinson's disease progression. *Brain Research* 1216, 78-86 (2008).

4.2 Book(s) and other one-time publications

Arbuthnott, G., Dejean, C., & Hyland, B. Antidromic cortical activity as the source of therapeutic actions of deep brain stimulation? *Cortico- subcortical dynamics in Parkinson's disease. ed Tseng, K.Y. Humana Press. New York* (2008).

Shindou, T., Arbuthnott, G.W., & Wickens, J.R. Neuromodulation and neurodynamics of striatal inhibitory networks: implications for Parkinson's disease. *Cortico-subcortical dynamics in Parkinson's disease. ed Tseng, K.Y. Humana Press. New York.* (2008).

Herrera-Marschitz, M., Arbuthnott, G.W., Ungerstedt, U. The rotational model and microdialysis: Significance for dopamine signallling, chlinical studies, and beyond., *Progress in Neurobiology*, doi: 10.1016/j.pneurobio.2009.01.005 (2009)

Arbuthnott, G.W., & Garcia-Munoz, M. Neuropharmacology *Chapter 3 in the Companion to Psychiatric Studies 8th Edition . Eds A. K. Zeally, E. Johnstone, C. Freeman. Churchill Livingstone, Edinburgh 2009* (2009).

Wickens, J.R., & Arbuthnott, G.W. Gating of Cortical Input to the Striatum. *Handbook of Basal Ganglia Structure and Function, a Decade of Progress. H. Steiner and K.S. Tseng, editors* (2009).

4.3 Oral presentations

Gordon, A. Brain structure and function, OCNC, June 16, 2008

Gordon, A. A Cortical source of the benefit of deep brain stimulation for parknsonism?, Univerisity of Oxford, UK, July 22, 2008

Gordon, A. A Cortical source of the benefit due to deep brain stimulation?, University of Edinburgh, UK, July 25, 2008

Gordon, A. Mechanism of deep brain stimulation in the treatment of movement of disorders., International Brain Research Organization School of Neuroscience, Hong Kong, January 8, 2009

Gordon, A. An unusual possibility for the mode of action of subthalamic stimulation in Parkinson's disease., Shiga University of Medical Science, Japan, February 20, 2009

Jolin Dahel, K., Stains, W.A., Schock, S. The role of striatal cholinergic interneurons., Shiga University of Medical Science, Japan, February 20, 2009

Schock, S. Uncovering the molecular mechnisms of preconditioning., Shiga University of Medical Science, Japan, February 20, 2009

4.4 Posters

Hyland, B., Dejean, C., Sheerin, A., Arbuthnott, G., Wickens, J. Gamma- and beta-oscillations in rat globus pallidus are differentially modulated during immobility induced by dopamine D2- or D1-receptor antagonists., FENS Meeting, Geneva, Switzerland, 12-16 July 2008, Biannual International Congress

5 Intellectual Property Rights and Other Specific Achievements

6 Meetings and Events

6.1 Seminar

Date: July 4th, 2008
Venue: IRP Conference Room
Speaker: Professor Paul Bolam
MRC Anatomical Neuropharmacology Unit
Department of Pharmacology, University of Oxford

6.2 Seminar

Date: October 9th, 2008
Venue: IRP Conference Room
Speaker: Fiona Randall, Ph.D.
Neural Networks Group, Institute of Neuroscience
Newcastle University

6.3 Seminar

Date: October 14th, 2008
Venue: IRP Conference Room
Speaker: Jan Schulz, PhD student
Department of Anatomy and Structural Biology
University of Otago

6.4 Seminar

Date: November 26th, 2008
Venue: IRP Conference Room
Speaker: Takuya Hikima, Ph.D.
Department of Development Biology and Neuroscience
Tohoku University Graduate School of Life Science

6.5 Seminar

Date: February 19th, 2009
Venue: IRP Conference Room
Speaker: Luis Alberto Carrillo-Reid, Ph.D.
Institute of Cell Physiology
Department of Biophysics and Neuroscience
National University of Mexico

6.6 Seminar

Date: February 26th and 27th, 2009
Venue: IRP Conference Room
Speaker: Dr. Stephan Theiss
Neurochip Laboratory
Department of Neurology
University of Dusseldorf

Molecular Genetic Unit

Principal Investigator:
Sydney Brenner

Research Theme:
Molecular genetics of salamander

Abstract

Salamander is thought to be a good material for neuroscience because of its large cell size and relatively simple nervous system. We previously constructed cDNA libraries of brain, retina, and spinal cord of the salamander (*Ambystoma mexicanum*). We carried out first-run sequencing of 5'-ends of about 200,000 clones from the libraries. These sequences were assembled into 16,195 clusters, which is almost equivalent to the number of cloned genes. In this fiscal year we completed the full-length sequencing of the representative clones from each cluster. We are now constructing a cDNA database of them.
During the year gene sequencing resources were pooled into a sequencing center, and next generation sequencing machines were obtained.

1 Staff

Technical Staff: Shoko Takehara
Saori Goda
Shin-ichi Yamasaki
Miho Hirai

2 Partner Organizations

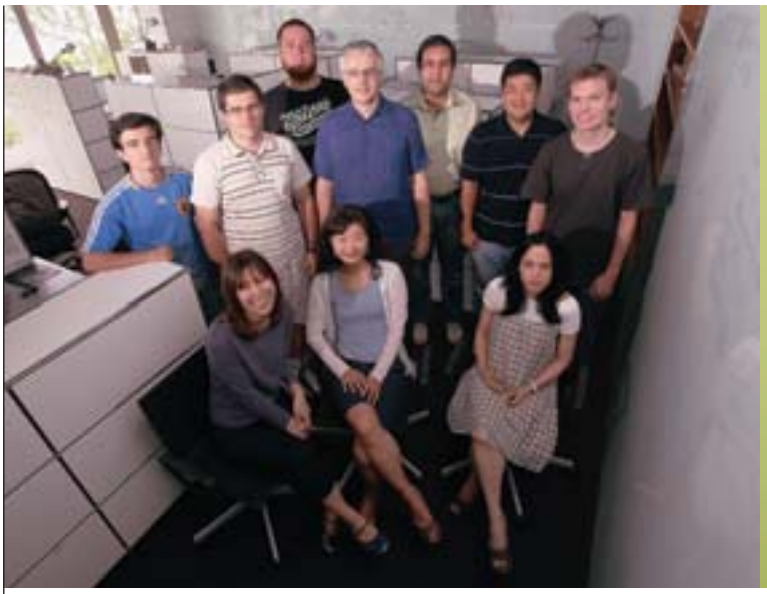
Okinawa Institute of Science and Technology PC
Type of partnership: Joint research
Name of principal researcher: Dr. Takayuki Naito
Name of researchers: Setsuko Nakanishi, Kiyotaka Akiyama, Seiko Kuraba
Research theme: Salamander project

3 Activities and Findings

3.1 Salamander project (See Naito unit.)

We completed full-length sequencing of 16,195 cDNAs from cDNA libraries of brain, retina, and spinal cord of the salamander (*Ambystoma mexicanum*). We are now constructing a cDNA database of them.
During the course of the year, it was decided to pool all of the sequencing resources and set up a sequencing center for use by all OIST researchers. Dr. Brenner has diverted his budget and technical support to the center. By the end of the year we had acquired next generation sequencing machines and had initiated several projects (Sato, Yanagida, Brenner). From next year, the Brenner unit will report as part of the sequencing center.

Computational Neuroscience Unit



Principal Investigator:
Erik De Schutter

Research Theme:
Modeling Cellular and Molecular Mechanisms of Neural Information Processing

Abstract

During the second year several new members joined the unit and started novel projects. These include molecular modeling of the effect of NO on induction of cerebellar long-term depression, studies of the planar structure of neuronal dendrites, modeling of the effect of ethanol on Golgi cell excitability and development of phenomenological models for cerebellar synaptic plasticity. In addition we started building a large-scale network of the system. We continued work on description languages for neural modeling and on the development of the Neurofitter and STEPS software packages. Several researchers made good progress developing both simple and more complex models of the Purkinje cell, with emphasis on the role of calcium dynamics in its dendritic excitability. We studied neuronal correlation in a class of neurons that are sensitive to rapid fluctuations in their input and discovered that in this case correlation is not dependent on neuronal gain.

1 Staff

General services and neuroinformatics
Technical Staff: Ivan Raikov
Research Administrator / Secretary: Tsuyuki Nakabayashi

Molecular modeling
Researcher: Gabriela Antunes (from January 2009)
Technical Staff: Iain Hepburn (from August 2008)
Stefan Wils (till September 2008)

Cellular modeling
Researchers: Sungho Hong
Yihwa Kim
Technical Staff: Haroon Anwar
Werner Van Geit

Network modeling
Researchers: Rodrigo Publio (from August 2008)
Thomas Sangrey

2 Partner Organizations

University of Antwerp, Belgium
Type of partnership: Scientific collaboration and graduate program
Name of principal researchers: M. Giugliano, D. Snyders

Name of researchers: K. Bhuvanasundaram, R. Maex, Q. Robberecht, K. Tahon, K. Veys
Research theme: Cerebellar physiology, multiple themes

ATR, Japan
Type of partnership: Scientific collaboration
Name of principal researcher: M. Kawato
Name of researcher: H. Ogasawara
Research theme: Molecular modeling of cerebellar signaling pathways

PRESTO, University of Tokyo, Japan
Type of partnership: Scientific collaboration
Name of principal researcher: K. Tanaka
Research theme: Molecular modeling of cerebellar signaling pathways

Tampere University of Technology, Finland
Type of partnership: Scientific collaboration
Name of principal researcher: M.-L. Linne
Name of researcher: K. Hituri
Research theme: Stochastic modeling of IP3 receptors

Duke University, United States of America
Type of partnership: Scientific collaboration
Name of principal researcher: G. J. Augustine
Research theme: Molecular modeling of diffusion in dendrites

University Texas, San Antonio, United States of America
Type of partnership: Scientific collaboration
Name of principal researcher: F. Santamaria
Research theme: Molecular modeling of diffusion in dendrites

University California, San Diego, United States of America
Type of partnership: Scientific collaboration
Name of principal researchers: M. Ellisman and M. Martone
Name of researcher: L. Fong
Research theme: Ultrastructural morphology of Purkinje cells

University of New Mexico
Type of partnership: Scientific collaboration
Name of principal researcher: C.F. Valenzuela
Research theme: Modeling of cerebellar Golgi neurons

University College London, United Kingdom
Type of partnership: Scientific collaboration
Name of principal researcher: M. Häusser
Name of researchers: H. Cuntz, A. Roth
Research theme: Purkinje cell morphology and physiology, modeling

Emory University, United States of America
Type of partnership: Scientific collaboration
Name of principal researcher: D. Jaeger
Name of researcher: N. Schultheiss
Research theme: Modeling of deep cerebellar nuclei neurons

3 Activities and Findings

3.1 Neuroinformatics standards

3.1.1 Languages for neuroscience modeling

As computational models of neurons become more complex, their software implementations become more difficult to define, comprehend, and communicate. Consequently, it would be very useful to have software tools that support easy exchange and understanding of computational models.

During the past year, we investigated the use of NeuroML (Crook and Howell 2007), a mark-up language for neuroscience, which is intended as a common format for exchange between neuron modeling software. We found major shortcomings with the existing standard, and communicated these to the standard developers. A report with our experiences using NeuroML was delivered during the Computational Neuroscience 2008 meeting, as part of the workshop on interoperability of neuroscience software. Several improvements have been made to NeuroML to address these issues since, but we believe that a more comprehensive approach is necessary. Hence, we have proposed an alternative approach, compatible with NeuroML, which we call the layer-oriented standard.

As a prototype implementation we have developed a language for describing models of ion channels that can be extended with arbitrary reaction kinetics and rate laws in order to model biophysical processes that affect ion channel dynamics. The elements of our prototype language are divided in two semantic layers, the descriptive layer and the abstraction layer. The descriptive layer consists of elements that correspond to biological modeling concepts, such as gates and ionic conductances. The abstraction layer consists of elements that correspond to general mathematical concepts, such as rate equations and functions. All elements in the descriptive layer are defined in terms of a combination of elements from the abstraction layer.

This prototype language is a step in the direction of formalizing the process of generating numerical simulation code from a high-level model specification, independent of particular software environments. Explicitly formalized semantic steps can contribute greatly to the expressiveness of modeling languages and, consequently, scientific understanding.

The currently implemented software to support this language includes a library for parsing and analysis of model descriptions and libraries for generating simulation code for the NEURON neural simulation environment and for the GNU Octave numerical computing environment.

3.2 Molecular mechanisms of synaptic plasticity

3.2.1 Software development of STEPS

STEPS is a software system for simulation of biological reaction- diffusion systems in well-mixed systems or complex 3D morphologies, such as the signaling pathways involved in synaptic plasticity. This year we have further developed and expanded STEPS.

STEPS implements a well-mixed solver based on the Gillespie Direct method (Gillespie 1976), capturing the stochastic nature of the reaction kinetics. Whilst this solver allows for investigation of the effects of noise in low concentration systems, the user may wish to choose a deterministic solution for systems of higher concentrations. For this reason we have added a deterministic solver for well-mixed systems which implements an algorithm based on the fourth-order Runge-Kutta method to approximate solutions to the classical chemical kinetics equations over a user-defined time step. If the user wishes to switch between solvers to investigate the solution of the model by different methods, minimal changes are required to the Python scripts.

STEPS includes a third solver which extends the SSA for simulating diffusion between voxels in a 3D tetrahedral mesh (Gillespie 1996). A paper describing how the SSA implementation was adapted for diffusion in STEPS is in preparation. An important innovation, which is in its final stages, is the extension of this reaction-diffusion modeling with simulation of the electrical membrane potential. This is important to enable the user to do a complete simulation of the interaction of voltage-gated channels in the cell membrane with the signaling pathways inside, allowing for bilateral interaction - e.g. voltage-gated calcium influx and gating of channels by calcium concentration. We analyzed different possible scenarios, including integration with a specialized solver for electrophysiological models, like GENESIS or NEURON, and full simulation of the electrodiffusion equation (Lopreore et al. 2008). The first solution seemed potential numerically unstable while the second was too complex and computationally intensive. Therefore, in collaboration with Robert Cannon (Textensor Inc., UK), an approximate method was developed in FY2007 that computes the effect of membrane currents on

membrane potential and axial spread of currents in the same 3D mesh used for the reaction-diffusion simulation, but neglects the effect of the weak electrical fields on diffusion of charged molecules. Currently we are validating this implementation and preparing a publication. In a later stage we will program APIs that allow STEPS to compute a small part of neuron model, while the voltage equation for the rest of the cell is solved by GENESIS or NEURON.

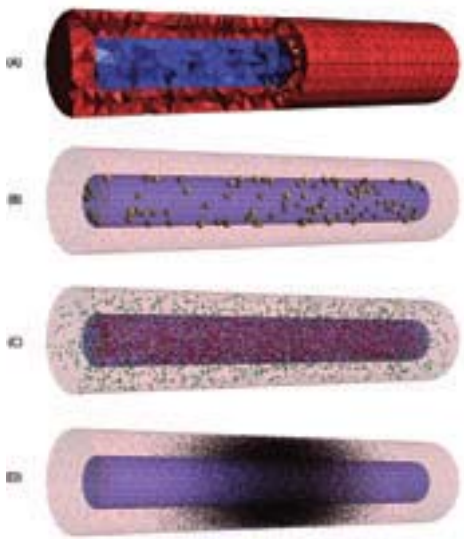


Figure 1. Flexible specification of initial conditions in STEPS. A: This opaque view with a cut-out shows that 21,090 tetrahedons are used to describe one cylinder surrounding another one. **B:** Membrane channels C are distributed randomly over the inner membrane (one line of Python code). **C:** A uniform initial distribution for molecules A and B (two lines of Python code). **D:** A Gaussian distribution in the center of the outer cylinder for X (12 lines of Python code).

Finally, we have made significant changes to the internal structure of STEPS that involved a complete review and re-write of the code. Previously we decided to integrate STEPS with the Python scripting language by reordering part of the code into Python extension modules (Wils and De Schutter, in press). This greatly simplified setting up the model and its initial conditions (Figure 1). This year we have improved on the initial restructuring by fully re-writing the most recent version of STEPS in C++, whilst great effort has gone into ensuring the user-interface in the automatically-generated Python modules remains compatible with previous developer-written versions. When reviewing the code we have made considerable improvements resulting in software that is more reliable to use, such as more thorough argument checking and more robust exception-handling to catch any user-initiated error. We have also expanded the user interface by adding more methods to the model, geometry and solver Python objects to allow the user to extract more information from their model and to have greater control of their simulation

3.2.2 Stochastic modeling of the signaling pathways involved in cerebellar LTD

Long-term depression (LTD) is a persistent decrease in the efficacy of synaptic transmission that results from the reduction of the number of AMPA receptors (AMPARs) in the postsynaptic cellular membrane (Linden, 2001; Derkach et al., 2007) and is important in learning and memory (Boyden et al., 2004).

We are using the STEPS software to develop a realistic, stochastic biochemical model of the cerebellar LTD to gain insights in its biochemical and cellular mechanisms. We started our work focusing on previous data that reported that, in Purkinje cells, LTD can be induced just by increasing the postsynaptic calcium concentration ([Ca^{2+}]) by flash photolysis of caged Ca^{2+} (Tanaka et al., 2007). To simulate this process, we extending existing models (Kuroda et al., 2001, Doi et al., 2005) of the principal pathways involved in cerebellar LTD (Figure 2A). An important innovation is that we succeeded, for the first time, to simulate these models in stochastic mode. In addition several reactions were updated conforming to recent literature. The initial version of the model is well-mixed. After developing this model, we simulated the flash photolysis of caged Ca^{2+} to verify the occurrence of LTD. In accordance with prior work, our initial results indicate that there is a correlation between the magnitude of LTD, verified through the number of phosphorylated AMPARs, and the amplitude of

the postsynaptic [Ca^{2+}] (Figure 2B).
Subsequently, we started expanding our model as a part of collaboration with Keiko Tanaka and George Augustine. Based on their experimental data, our main objective is to use our computational model to study the regulatory role of the nitric oxide (NO)- cGMP-dependent protein kinase (PKG) pathway on LTD induction, focusing on the action of this pathway upstream of the Ca^{2+} signals in Purkinje cells. The NO-PKG pathway has two targets in our model: the inositol 1,4,5-trisphosphate receptor (IP_3R) and G-substrate, and our goal is to investigate whether the action of PKG on those molecules can decrease the half-maximum [Ca^{2+}] necessary to induce LTD. At this point, we already expanded the initial model to include the NO-PKG pathway and some other molecules and receptors (Figure 2C).

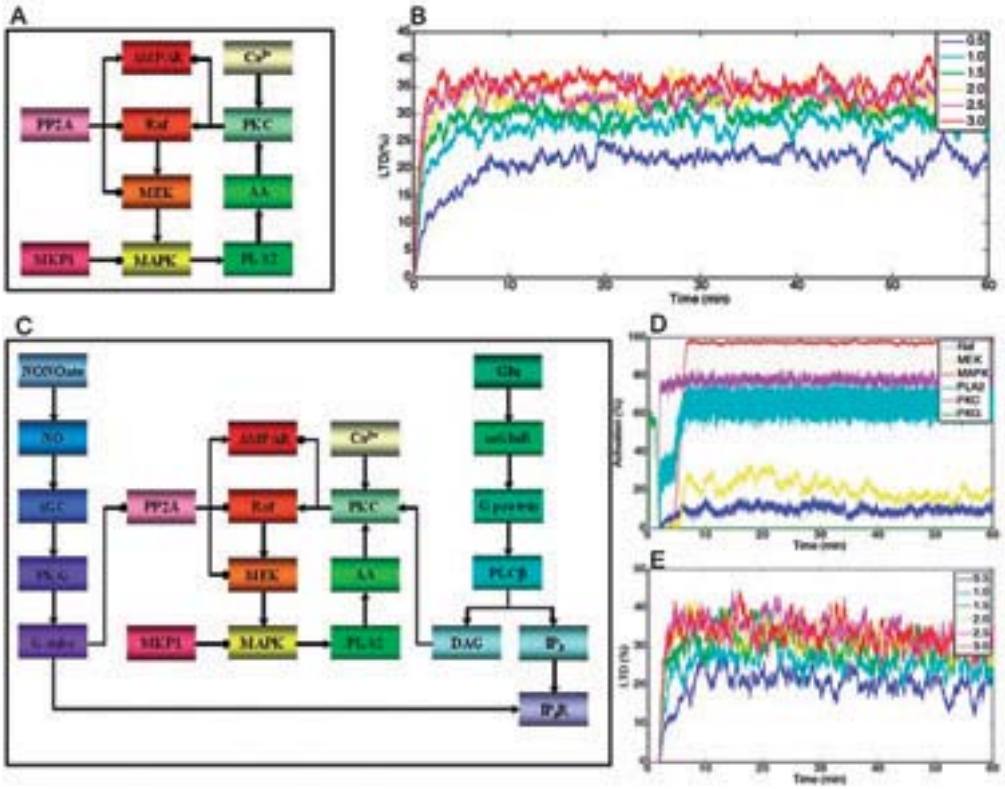


Figure 2. Signaling pathways model of cerebellar LTD. A: block diagram of the biochemical pathways used to simulate the cerebellar LTD induced by flash photolysis of caged Ca^{2+}. **B:** percentage of LTD obtained with our model stimulated by different postsynaptic [Ca^{2+}] (0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mM). **C:** block diagram of the biochemical network that has been used to study the role of NO-PKG pathway in the Ca^{2+} induced LTD. **D:** some results obtained with our expanded model of cerebellar LTD showing the activation of Raf, MEK, MAPK, PKC, PLA2 and PKG induced by NONOate, a NO donor, and 3 mM of [Ca^{2+}] applied 2 minutes after the NONOate. **E:** magnitude of LTD induced by different [Ca^{2+}] in presence of 10 mM of NONOate.
Abbreviations: AMPAR, AMPA receptor; MKP1, MAP kinase phosphatase 1; AA, arachidonic acid; Raf, Raf kinase; MEK, MAPK/ERK kinase; MAPK, mitogen-activated protein kinase; PLA2, phospholipase A2; AA, arachidonic acid; PKC, conventional protein kinase C; Glu, L-glutamate; mGluR, metabotropic glutamate receptor; PLCb, phospholipase C beta; DAG, diacylglycerol, IP_3, inositol 1,4,5-trisphosphate, IP_3R, inositol 1,4,5-trisphosphate receptor; NONOate, 1,1-diethyl-2-hydroxy-2-nitrosohydrazine; sGC, soluble guanylate cyclase; NO, nitric oxide; PKG, cGMP-dependent protein kinase, G-subs, G-substrate.

Consistently with our previous results, the simulations performed with the expanded model indicate a correlation between the magnitude of LTD and the postsynaptic [Ca^{2+}] (Fig. 1E). Now, we are improving the Ca^{2+} dynamics of our model by adding buffers, pumps and diffusion.

3.2.3 Anomalous diffusion in spiny dendrites

As reported last year we have extended our combined modeling-experimental study on anomalous diffusion in Purkinje cell spiny dendrites (Santamaria et al. 2007) to spiny dendrites of hippocampal pyramidal neurons; a collaboration with Fidel Santamaria and George Augustine. In doing so we

confirmed the relationship between spine density and the degree of anomaly of diffusion, but also were under the impression that this relationship was stronger in pyramidal neurons than in Purkinje cells. We have recently reassessed this observation based on a recent report (Vecellio et al. 2000) that found a much lower density of spines in Purkinje cells than has previously been reported using EM (Harris and Stevens 1988). This has led to a revision of the paper describing our results. We now conclude that there are no differences between the two cell types but that in both neuron types diffusion is more anomalous than expected for trapping by spines only. To further investigate possible mechanisms of the increased anomalous diffusion we have initiated a collaboration with Mark Ellisman and Maryann Martone to measure Purkinje cell spine density and composition using modern ultrastructural methods.

3.3 Cellular mechanisms regulating firing and synaptic properties of neurons

3.3.1 Software development of Neurofitter

Neurofitter (Van Geit et al. 2007), a software tool developed in our group, automates the process of fitting neuron model parameters. Its purpose is to find parameter values so that the output generated by a computational model that uses these values shows the same voltage response to specific inputs (e.g. different current steps) as obtained from experiments. The software provides the user with a number of search methods that optimize an error function (Figure 3) based on a comparison between phase plane trajectory density plots of experimental data and model output.

We made improvements to the method to generate better model parameter sets. These include the implementation of another search algorithm called NSGA2 (Deb 2002), which is an evolutionary search algorithm that has several advantages. It is multi-objective, meaning that the optimization doesn't make use of one single value to measure the fitness of a possible solution. In that way it is able to optimize different objectives separately. Another advantage is the 'sharing function' that the algorithm uses. It prevents solutions from becoming clustered around local minima since it pushes search paths that come close together apart, causing the search to become more evenly spread in the solution space.

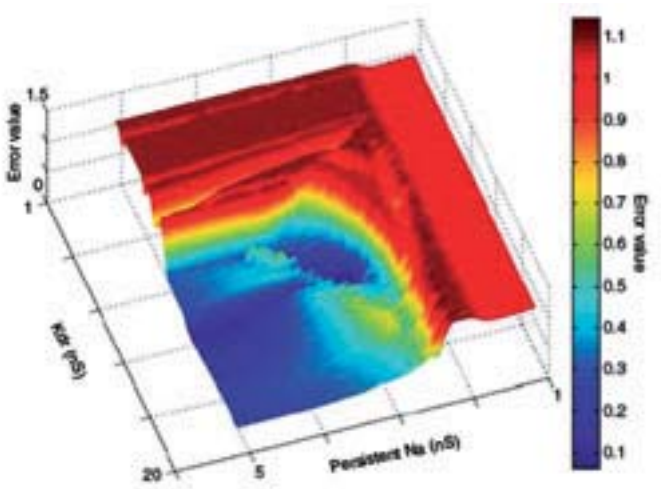


Figure 3. 3D plot of error function used by Neurofitter. The Z-axis shows the error value for a single-compartmental model run with the corresponding maximal conductance values (for persistent Na and delayed rectifier K ion channels) on the X- and Y-axes.

Another improvement consisted of releasing more model parameters. Previously the only free parameters in the search were the maximal conductances of the ion channels, together with parameters for the passive structure of the model like membrane resistance, axial conductance, etc. This, however, created too many constraints for the search method. There were several fixed parameters that were not tuned by the search method, like voltage-dependence and time constants of the ion channels kinetics. If these fixed parameters are too different from those in the cell where the actual experimental data was recorded (and for which the values are unknown), it becomes impossible for any combination of the tuned parameters to generate output that corresponds to the experimental data. We decided to release many of the kinetic parameters for voltage-gated channels (originally we were using values published in Akemann and Knöpfel 2006), so that they became part of the search process itself. This meant a significant increase in the number of search parameters. As

an example, the one-compartmental Purkinje cell model we are fitting currently has about 100 free parameters. The search method, however, converges to solutions that show action potential shapes that are significantly closer to the experimental data than the results we obtained before.

3.3.2 Soma-dendrite interaction in a simple Purkinje neuron model

Cerebellar Purkinje neurons have an enormously elaborate dendritic tree, and its role in information processing has been extensively discussed (Hong and De Schutter 2008). The dendrites are equipped with active mechanisms including voltage dependent Ca^{2+} and Ca^{2+}-activated K^+ currents. Therefore, even though a somatic spike barely propagates into the dendritic tree (Vetter et al. 2001), there is interaction between soma and dendrite, which enables the dendritic tree to control the spiking patterns both in absence (Womack and Khodakhah 2004) and presence of synaptic input (Davie et al. 2008; McKay et al. 2007).

Among the diverse active mechanisms, we focused on the P/Q-type Ca^{2+} channel and large conductance Ca^{2+}-dependent K^+ (BK) channel since they are the most important currents governing the fast Ca^{2+}-dependent activity. We constructed a Hodgkin-Huxley type model of the P/Q-type Ca^{2+} channel from experimental data provided by Bruce Bean (Harvard University, USA), and similarly a BK channel model from published data. For other channels such as T-type Ca^{2+} channel and small conductance K^+ (SK) channel, we used the models from Solinas et al. (2007).

These mechanisms were inserted into a simple morphology composed of three compartments, which represent soma, smooth dendrites, and spiny dendrites. This choice was primarily made to see if the channel mechanisms can reproduce the qualitative patterns of soma-dendritic interactions during activity. Parameter tuning was carried out either by hand or using Neurofitter (Van Geit et al. 2007).

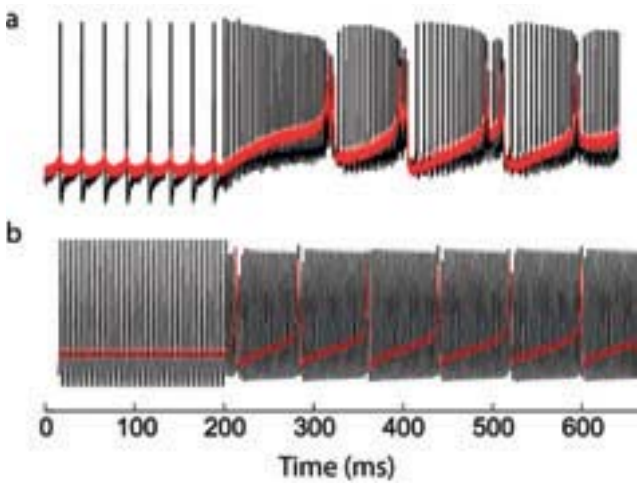


Figure 4. Interaction of the soma and dendritic tree in a real and model Purkinje neuron. **a:** Experimental data from Arnd Roth. The black and red traces are somatic and dendritic recordings, respectively. **b:** Model Purkinje neuron with three compartments shows qualitatively similar behavior.

The model indeed shows qualitative features resembling the data (Figure 4): the dendritic compartment can generate Ca^{2+}-dependent spikes, which are driven by the Na^+-dependent spikes from the soma. This shows that even though the somatic influence of the Na^+ spikes on the dendritic tree can be very small, it might be able to interact with the dendritic Ca^{2+} mechanisms when sufficiently high spiking frequency is sustained.

3.3.3 Modeling detailed calcium dynamics in the Purkinje cell

An important step towards building a new Purkinje cell model is to improve the representation of calcium dynamics necessary for the activation of Ca^{2+}-activated K^+ channels. Recently, it has been shown that BK channels are in closer vicinity of Ca^{2+} sources as compared to SK channels (Falker et al. 2008), suggesting that activation of BK channels require a brief large amount (about 10 – 100 μM) of Ca^{2+} whereas SK channels require longer activation by smaller quantities (about 0.1 – 2 μM) of Ca^{2+}. Previous models of BK channels used in recent PC models (Khaliq et al. 2003, Akemann et al. 2006) are not properly modulated by changes in internal Ca^{2+} concentration. Therefore, we developed new equations for the BK channels, using the kinetic scheme proposed by Moczydlowski and Latorre

(1983) and tuning the parameters to fit to recent experimental data (Sun et al. 2004).
Using these equations we are investigating whether a fairly detailed model of well-mixed Ca^{2+} dynamics with several buffers and pumps (Schmidt et al. 2003) suffices to activate the BK and SK channels correctly. This would have the advantage that we avoid the need of computationally more intensive spatial models of Ca^{2+} dynamics (including diffusion) in large compartmental neuron models, while still capturing the temporal properties of the biological system based on the real biophysics.

3.3.4 Local planar structure of dendritic trees

Global planar structures are characteristics of 'flat' neurons like Purkinje cells and retinal ganglion cells. Furthermore, based on measuring the cone angle between parent and daughter branches, it has been proposed that local planar dendritic structures are present in pyramidal neurons of the rabbit visual cortex (Uylings and Smit 1974). These authors found that the cone angle distribution was skewed towards angles of 180°.
We used a numerically more stable way of computing cone angles, developed by Dr. R. Sinclair of OIST, to confirm this observation by measuring cone angles in 8 different types of neurons (8 to 30 cells for each type), downloaded from the NeuroMorpho.org database.
Next we evaluated whether this observation had special significance for biological dendrites by generating random cone angle distributions using Monte Carlo simulation. Points were first randomly distributed in a sphere. We included a large number of points in the sphere and chose points close to the center to avoid boundary effects. First the point closest to the center (B: branch point) was identified. Next, three points closest to point B were taken (P, A, C). When the branch point (B) was connected to these three closest points (BP, BA, BC), we discovered that the local connection length was minimized in 80% of the cases. Furthermore, in this case the cone angle distribution, with B as the tip of the cone, results in a distribution similar to that observed in biology (Figure 5). In contrast, when the four points (B, P, A, C) were connected in a random manner, the resulting distribution had its mean close to 90° (Figure 5). In summary, the Monte Carlo modeling predicts that if dendritic branches connect to the closest points in space, local dendritic length is minimized and the resulting cone angle will be close to 180°.
Therefore, no additional mechanisms are needed to explain the observed cone angle distributions, such as tension or forced equilibria between the parent and daughter segments (Uylings and Van Pelt 2002). Instead cone angle distributions are the predicted consequence of a simple connection length optimization during dendritic growth.

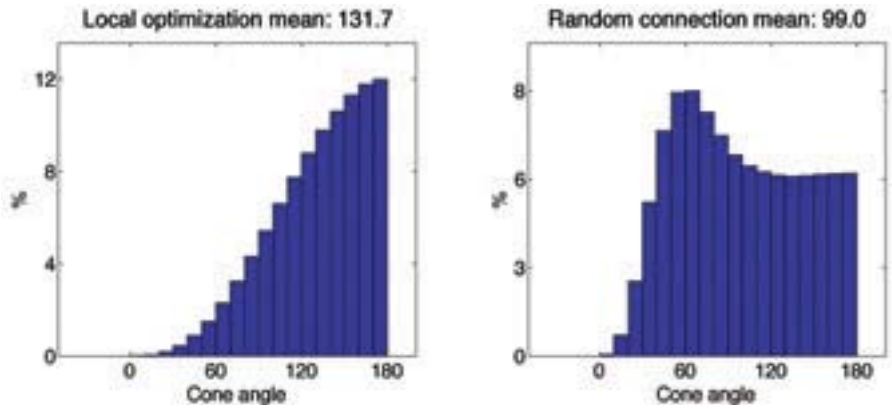


Figure 5: Distribution of cone angles, predicted by Monte Carlo simulation.
Left: when local connection length is minimized, the distribution is similar to the one observed in biology. **Right:** when connections are made randomly the resulting mean is close to 90 degrees.

3.3.5 Implications of rich single neuron computation for correlation of population activity

Pair wise correlation is a widely observed neural phenomenon in population activity. In particular, even with the same mean stimulus, noisy fluctuations of population firing are often correlated. This noise correlation has attracted a lot of attention in regard to whether it might transfer independent information beyond a mean population response (Averbeck, Latham, & Pouget, 2006). However, in

the context of the common input model, where common input noise drives the noise correlation, a recent influential study suggested that the noise correlation must have a simple relationship to the mean firing rate, or more precisely the mean gain (Figure 6a-c, de la Rocha et al. 2007). The authors therefore claim that noise correlation may not carry any independent information.

We have verified this hypothesis by using computer simulations of various neuron models. In some models, we found that the correlation-gain relationship fails to capture a large portion of the actual correlation (Figure 6d-e). Our further analysis revealed that this failure of the relationship tends to coincide with the frequent occurrence of synchronized spikes. Considering that the correlation-gain relationship is based on the assumption that neural correlation originates from the comodulation of firing rates, noise-induced synchronization can be an explanation for the additional correlation observed.

We showed that the synchronized spikes can easily be generated when the neuron's gain (with respect to the stimulus mean) is positively modulated by the amount of additive noise (variance). This has been observed in the avian brain stem, layer 2/3 of prefrontal cortex (Higgs et al. 2006), and layer 5 of medial-prefrontal cortex (Arsiero et al. 2007). This particular property can be implemented by diverse means such as the type 3 membrane excitability (Lundstrom et al. 2008), long-term activation of the outward current (Higgs et al. 2006), slow inactivation of the inward current (Arsiero et al. 2007), etc.

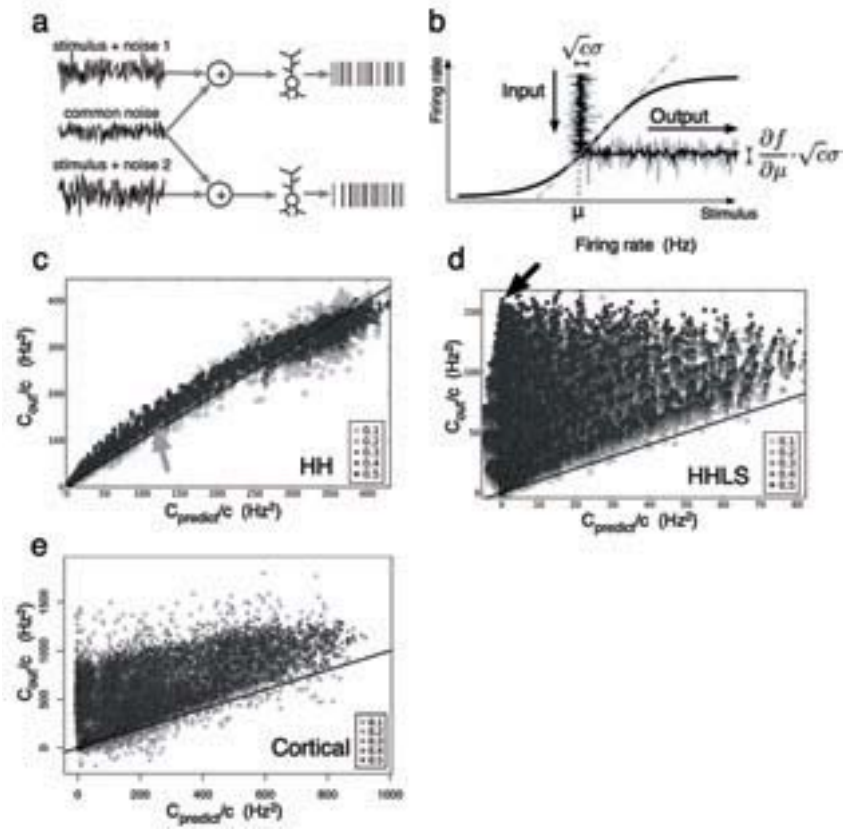


Figure 6. Relation between correlation and gain in different models. a: Schematic of the common input model. **b:** Linear perturbation in firing due to noise (de la Rocha et al., 2007). The common noise (black, vertical) in the total input (gray, vertical) induces the correlated component (black, horizontal) of the total firing rate fluctuation (gray, horizontal), which can be linearly approximated with the gain (gray dotted). **c, d, e:** A ratio of the output covariance and input correlation vs. prediction of the correlation-gain relationship in HH (c), HHLS (d), and cortical pyramid neuron models (e). A large shunting conductance is added to the cortical model to mimic the *in vivo*-like condition (Prescott et al. 2006).

However, the common result is that the neuron develops sensitivity to rapid fluctuations in the stimulus, and therefore shows higher gain when the stimulus noise has higher variance. By using a simple functional model, composed of a filter and threshold, we were able to show that this property leads to a large correlation coming from synchronized spikes.

We conclude that single neurons can process the information carried by dynamic stimuli using a surprisingly rich coding strategy, and therefore the question arises how the output of such neurons is

decoded. The correlation-gain relationship indicates that the only relevant variable is the mean firing rate (de la Rocha et al., 2007), but our result shows that rich single neuron computation may also lead to a rich structures of the population activity, which cannot be solely measured as the mean firing rate. We are at present trying to get experimental verification of our results before submitting a paper.

3.4 Information processing in the olivocerebellar system

3.4.1 Cerebellar network modeling

The development of a large-scale network model of the cerebellum has been started. The model includes multiple classes of conductance-based neuron models that have been matched to physiological behavior and are connected in a way that realistically reflects cerebellar anatomy. The granular cell layer is the primary input processing stage of the cerebellum and our simplified rendering of this structure is accomplished by connecting two major cell populations: the granule cells (D'Angelo et al, 2001) and Golgi cells (Solinas et al, 2007) in a ratio of about 1000 to 1. Mossy fibers, the major input stream to the granule cell layer are modeled as an array of artificially spiking neurons with no correlation but a fixed average firing rate and Poisson generated spike events.
The network improves on the one by Maex and De Schutter (1998) by capturing more realistic aspects of cerebellar anatomy in 3D. Synaptic connections between cell populations occur within the network setup by defining a set of rules that incorporate connectivity range, probability, and anisotropy. For example, the parallel fiber beams can realistically make synapses with any Golgi cell in its path (1-d anisotropy) with a fixed probability that falls off to zero outside of 90 μM width transverse to the parallel fiber (PF) beam path. Similar scenarios can be used among each pair of cell populations (including MF inputs) to create variant network topologies for exploration.

3.4.2 Intrinsic mechanisms underlying ethanol induced excitability of Golgi cells

Golgi cell baseline firing has been shown to increase in the presence of ethanol (Botta and Valenzuela, in preparation). This has consequences upon the granule cell layer activity because it has been shown that granule cell layer oscillations are entrained to Golgi cell activity (Maex and De Schutter, 1998). In Figure 7, the effects of ethanol upon Golgi cell firing behavior is summarized.

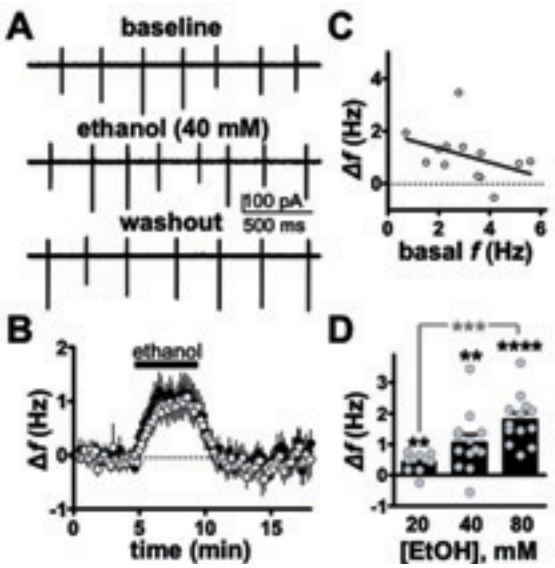


Figure 7: Ethanol increases Golgi cell excitability (from Botta et al, in preparation). A, B) in slice preparation loose cell attached recordings in the presence of synaptic blockers reveals ethanol induced excitability which is not significantly correlated with baseline firing rate (C). D) Dose response of Golgi excitability upon ethanol concentration.

In an ongoing collaboration with Fernando Valenzuela, we have investigated with single cell modeling what intrinsic conductance mechanism could be the possible target of ethanol-induced excitability in Golgi cells. Plausible candidates that have emerged based on a systematic parameter sensitivity study are the fast transient potassium current, I_A, the non-inactivating potassium current, I_m, and the Na/K hyperpolarizing pump current. As a response to our modeling studies, the effect of these currents will be studied using pharmacological methods by the Valenzuela group. Using a

plausible model of ethanol excitability, the impact of this effect upon granule layer network function will then be explored and quantified.

3.4.3 Phenomenological models of Purkinje cell short-term and long-term plasticity

The Purkinje cell provides the only output of the cerebellar cortex and receives excitatory input from PFs (~200,000 axons) and strong excitation from a single climbing fiber (CF). Recent experimental works shows that both synapses express multiple forms of plasticity, where the synaptic weight changes according presynaptic firing rates or intracellular calcium concentration (Hansel et al., 2001; Jörntell and Hansel, 2006). Since synaptic plasticity is considered one of the main mechanisms involved in motor learning, it is essential to understand how these mechanisms interact with each other under different input conditions.

We are building phenomenological models for the PF and CF synapses based on previous approaches (Abbott et al., 1997; Migliore and Lansky, 1999) to mimic the postsynaptic forms of long-term and short-term plasticity observed experimentally. While detailed models are essential to understand the signaling mechanisms underlying synaptic plasticity (section 3.2.2), phenomenological models that describe synaptic changes with more general equations can be good approximations and are less computationally expensive.

In our model we use a single compartment for the Purkinje cell (Akemann and Knöpfel, 2006). Both PF and CF synapse are connected to the single postsynaptic compartment, and the plasticity in both synapses is driven by the same postsynaptic calcium mechanism. The CF synapse includes one LTD mechanism described by a calcium dependent learning rule, generating an S-shape relationship between plasticity amplitude and intracellular calcium concentration that fit the experimental data well. The model responses are very robust to changes in the parameter space and in the number of events used during the training phase. When the synapse was stimulated with a periodic stimulus, it only induced depression for frequencies higher than 4Hz, in good agreement with experimental procedures (Hansel et al., 2001).

The PF synapse model was formulated in a way to include the postsynaptic form of LTD (Tanaka et al., 2007), long-term potentiation (LTP) and short-term facilitation. PF LTD is induced when the PF synapse receives massive input or if PF and CF are stimulated together at low frequency, while PF LTP is induced when the PF is stimulated at low frequency in the absence of CF input (Hansel et al., 2001). Our model reproduces the bidirectional long-term plasticity observed in the experimental data in agreement with an inverse BCM rule, where there is a higher calcium threshold for LTD than for LTP. At a smaller time scale, PF fiber synapses can also control the amplitude of evoked EPSPs depending on the incoming spike train. Each successive spike can evoke an EPSP with higher amplitude than the previous one due to short-term facilitation (Empson et al., 2008).

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4 Publications

4.1 Journals

Achard, P. & De Schutter, E. Calcium, synaptic plasticity and intrinsic homeostasis in Purkinje neuron models. *Frontiers in Computational Neuroscience* 2, 8 (2008).

De Schutter, E. Reviewing multi-disciplinary papers: a challenge in neuroscience? *Neuroinformatics* 6 (4), 253-255 (2008).

De Schutter, E. Why are computational neuroscience and systems biology so separate? *PLoS Computational Biology* 4 (5), e1000078 (2008).

De Schutter, E. & Steuber, V. Patterns and Pauses in Purkinje Cell Simple Spike Trains: Experiments, Modeling and Theory. *Neuroscience*, in press (2009).

Hong, S. & De Schutter, E. Purkinje neurons: What is the signal for complex spikes? *Current Biology* 18 (20), R969-971 (2008).

Van Geit, W., De Schutter, E. & Achard, P. Automated neuron model optimization techniques: a review. *Biological Cybernetics* 99 (4-5), 241-251 (2008).

4.2 Book(s) and other one-time publications

Nothing to report.

4.3 Oral presentations

Sangrey, Thomas D. Intrinsic currents underlying the rebound depolarization in deep cerebellar nucleus neurons, OIST Internal Seminar, June 13, 2008

De Schutter, Erik. Modeling Neurons: Building Connections between Europe and Japan, Tsukuba University, Tokyo, June 22, 2008

De Schutter, Erik. Computational Neuroscience and Systems Biology: the Past, the Now and the Future, CNS*2008 Meeting, Portland, Oregon, USA, July 21, 2008

De Schutter, Erik. Anomalous Intracellular Diffusion in Spiny Dendrites of Pyramidal Neurons and Purkinje Cells, CNS*2008 Workshops, Portland, Oregon, USA, July 23, 2008

Raikov, Ivan. Neuroscience modeling languages: practice and theory, CNS*2008 Workshops, Portland, Oregon, USA, July 23, 2008

Hong, Sungho., De Schuter, Erik. Correlation susceptibility and single neuron computation, OIST Junior Retreat, October 30, 2008

De Schutter, Erik. The Purkinje neuron model parameter space: implications for homeostasis and synaptic plasticity, EPSRC Symposium on Computational Neuroscience, Warwick, UK, December 8, 2008

De Schutter, Erik. Automated parameter searches for large single neuron models: Purkinje cell excitability and plasticity, The Winter Workshop 2009: Mechanism of Brain and Mind, Rusutsu, Hokkaido, Japan, January 14, 2009

Van Geit, Werner. Using Neurofitter to fit a Purkinje cell model to experimental data, OIST Internal Seminar, March 13, 2009

4.4 Posters

Veys, Ken., Raes, Adam., Snyders, Dirk., De Schutter, Erik. Correlating the expression profile with electrophysiology in a single Purkinje cell in acute brain slices, 6th Forum of European Neuroscience, Geneva, Switzerland, July 12-16, 2008

Robberechts, Quinten., Wijnants, Marc., De Schutter, Erik. Long-term depression at parallel fiber to Golgi cell synapses, 6th Forum of European Neuroscience, Geneva, Switzerland, July 12-16, 2008

Achard, Pablo., De Schutter, Erik. Calcium, synaptic plasticity and intrinsic homeostasis in Purkinje neuron models, 6th Forum of European Neuroscience, Geneva, Switzerland, July 12-16, 2008

Solinas, Sergio., Forti, Lia., Cesana, E., De Schutter, Erik. d'Angelo, Egidio. Fast-reset of pacemaking and theta-frequency resonance in cerebellar Golgi cells: simulations of their impact in vivo, 6th Forum of European Neuroscience, Geneva, Switzerland, July 12-16, 2008

Tahon, Koen., De Schutter, Erik., Maex, Reinoud. In vivo multi-electrode recordings in rat cerebellar cortex show a layered input pattern to Golgi cells during tactile stimulation, 6th Forum of European Neuroscience, Geneva, Switzerland, July 12-16, 2008

Van Geit, Werner., De Schutter, Erik. Using Neurofitter to fit a Purkinje cell model to experimental data, 6th Forum of European Neuroscience, Geneva, Switzerland, July 12-16, 2008

Wils, Stefan., De Schutter, Erik. Reaction-diffusion in complex 3D geometries: mesh construction and stochastic simulation with STEPS, 6th Forum of European Neuroscience, Geneva, Switzerland, July 12-16, 2008

Anwar, Haroon., Riachi,Imad., Hill, Sean L., Schürmann, Felix., Markram, Henry. Repairing 3D morphological models obtained from in vitro experiments, 6th Forum of European Neuroscience, Geneva, Switzerland, July 12-16, 2008

Hong, Sungho., De Schutter, Erik. Correlation susceptibility and single neuron computation, CNS*2008 Meeting, Portland, Oregon, USA, July 20-22, 2008

Hituri, Katri., Wils, Stefan., Linne, Marja-Leena., De Schutter, Erik. Validating models of the IP3 receptor: reproduction of experimental data with stochastic simulations, Neuroinformatics 2008 Meeting, Stockholm, Sweden, September 7-9, 2008

Van Geit, Werner., De Schutter, Erik. Using Neurofitter to fit a Purkinje cell model to experimental data Society for Neuroscience 2008 Meeting, Washington DC, USA, November 15-19, 2008

5 Intellectual Property Rights and Other Specific Achievements

Nothing to report.

6 Meetings and Events

6.1 Seminar: Models of synaptic plasticity and their computational consequences

Date: May 14, 2008
Venue: Seaside House
Speaker: Dr. T. Trappenberg (Dalhousie University, Canada)

6.2 Okinawa Computational Neuroscience Course 2008

Date: June 16 – July 4, 2008
Venue: Seaside House, OIST
Co-organizers: K. Doya, K. Stiefel, J. Wickens
Co-sponsors: Nara Institute of Science and Technology
Japanese Neural Network Society
Speakers: Arbuthnott, Gordon, OIST, Japan
Bell, Tony, UC Berkeley, USA
Bhalla, Upi, NCBS, Bangalore, India
Butera, Robert, Georgia Insitute of Technology, USA
Deneve, Sophie, Ecole Normale Supérieure, France

De Schutter, Erik, OIST, Japan
Destexhe, Alain, CNRS, France
Doya, Kenji, OIST, Japan
Fairhall, Adrienne, University Washington, USA
Gewaltig, Marc-Oliver, Honda RIE, Germany
Häusser, Michael, University College London, UK
Ishii, Shin, Nara Institute Science Technology, Japan
Koch, Christof, California Institute of Technology, USA
Li, Zhaoping, University College London, UK
Longtin, André, University of Ottawa, Canada
Stiefel, Klaus, OIST, Japan
Tsodyks, Misha, Weizmann Insititute, Israel
Wang, Xiao-Jing, Yale University, USA
Wickens, Jeff, OIST, Japan

6.3 Seminar: Decision and performance interactions in stroke recovery
Date: September 16, 2008
Venue: IRP
Speaker: Dr. N. Schweighofer (University of Southern California, USA)

6.4 Seminar: The Ca^{2+} Pump for Synapse Plasticity
Date: November 27, 2008
Venue: Seaside House
Speaker: Dr. R. M. Empson (University of Otago, New Zealand)

6.5 Seminar: Diffusional barriers in neurons: From synapses to dendrites
Date: February 25, 2009
Venue: IRP
Speaker: Dr. F. Santamaria (University Texas San Antonio, USA)

6.6 Seminar: On the nature of neuronal branching
Date: March 6, 2009
Venue: IRP
Speaker: Dr. H. Cuntz (University College London, UK)

Neural Computation Unit

Principal Investigator:
Kenji Doya

Research Theme:
A Computational Approach to Molecular Mechanisms of Mind

Abstract

The goal of Neural Computation Unit is to elucidate the mechanisms of animal behaviors and human minds at multiple levels; from actions and emotions via the networks and neurons to the molecules and genes. Our specific focus is on how the brain realizes reinforcement learning, in which an agent, biological or artificial, learns novel behaviors by exploration and reward feedback. We combine top-down, computational modeling and bottom-up, neurobiological experiments to achieve this goal. The major achievements of the three subgroups in this fiscal year 2008 are the following.

a) The Dynamical Systems Group continued to improve the Bayesian system identification methods for systems biology and the software tool, "LetItB," which can automatically estimate parameters of models defined in SBML (systems biology markup language) to fit experimental data. To understand the cellular mechanisms behind spike timing dependence of synaptic plasticity in cortico-striatal synapses, we constructed a compartmental model from our own imaging data and performed simulation of calcium dynamics. We also developed new reinforcement learning frameworks to cope with high-dimensional sensory inputs and to be implemented in LSI chips.

b) The Systems Neurobiology Group started a new functional brain imaging experiments to clarify the brain mechanisms for model-free and model-based reinforcement learning. Recording and analysis of basal ganglia neuron firing during a decision making task extended to dorsal parts of the cortico-striatal loop circuit. To clarify the role of serotonin in actions for delayed rewards, we started local drug injection in addition to chemical measurement using micro dialysis.

c) The Adaptive Systems Group continued simulation and experiment using a colony of robots called "Cyber Rodents." We realized distributed evolution of reward functions and meta-parameters for reinforcement learning algorithms and analyzed how their settings relate to each other. We developed a new four-leg robot platform and started experiments of locomotion and vision-based navigation.

1 Staff

Dynamical Systems Group
Researcher: Junichiro Yoshimoto
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Takumi Kamioka
Mikihiro Kobayashi

Research Administrators / Secretaries: Emiko Asato
Chikako Uehara

2 Partner Organizations

ATR Computational Neuroscience Laboratories
Type of partnership: Joint Research
Name of principal researcher: Dr. Mitsuo Kawato
Name of researcher: Kenji Doya
Research theme: Functional brain imaging study of molecular basis of mind

Honda Research Institute Japan Co., Ltd.
Type of partnership: Joint Research
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Research theme: Biological modeling of basal ganglia in behavioral learning

Honda R & D Co., Ltd.
Type of partnership: Joint Research
Name of principal researcher: Masato Hoshino
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Makoto Ito
Katsuhiko Miyazaki
Kayoko Miyazaki
Research theme: Brain-like principle of flexible and autonomous behavior acquisition

NEC Corporation
Type of partnership: Contract Research
Name of principal researcher: Nobuki Kajihara
Name of researchers: Kenji Doya
Shinji Kimura
Eiji Uchibe
Junichiro Yoshimoto
Research theme: A feasibility study of IP core implementation of reinforcement learning

3 Activities and Findings

3.1 Dynamical Systems Group

SBML-compatible GUI toolkit for Bayesian system identification of biological networks [Yoshimoto, Inoue]

Bayesian system identification paradigm has a potential to provide coherent solutions to a hierarchy of problems in biological modeling, namely, i) hidden variable estimation, ii) parameter search, iii) model selection, and iv) experiment design. Marked features are that the "solution" is not a point, but

a probability distribution, and that the assumptions of a modeler are made explicit in a form of prior distribution.
We integrated the algorithms we had devised so far into a general-purpose parameter estimation tool for models described by SBML (system biology markup language), which provided the parameter estimation engine with a graphic user interface for easy operation by biologists. The took kit was released in April 2008 via www (http://www.nc.irp.oist.jp/software/).

Timing-dependent calcium responses model in striatum [Nakano, Yoshimoto]
The striatum is the input nucleus of the basal ganglia, and receives glutamate input from the cortex and dopamine input from the substantia nigra. Recently, several studies reported contradictory results of its timing dependence on the cortical and dopamine inputs and the spike output. To clarify the mechanisms behind spike timing-dependent plasticity of striatal synapses, we investigated the spike timing-dependence of intracellular calcium concentration by constructing a multi-compartment striatal neuron model with realistic morphology. Our simulation predicted that the calcium transient is maximal when cortical spike input and dopamine input preceded the postsynaptic spike. The gain of the calcium transient is enhanced during the "up-state" of striatal cells.
Furthermore, we set up an electrophysiological recording platform to test the validity of our striatum signaling cascade model with the help of Wickens Unit. In this fiscal year, we distinguished the cell types of medium spiny neurons using eGFP transgenic mice, and investigated the long-term synaptic plasticity of the different cell types. Then, we explored a protocol for real-time recording of intracellular calcium transient using caged calcium.

Efficient spatio-temporal representation in reinforcement learning tasks with high-dimensional sensory inputs [Otsuka, Yoshimoto]
Reinforcement learning (RL) is a very attractive framework to build decision-making function into autonomous agents, and many methods for solving RL problems (e.g. Q-learning, SARSA, Actor-Critic, policy gradient, etc.) have been proposed so far. These methods have potential to solve any problem formulated as a Markov decision process (MDP), but many real problems do not fall into the class of MDP due to the presence of hidden states and the limitation of computational resources in the agents. The key for enhancing the performance of the RL methods in such problems is to extract spatiotemporal features lying behind the environment. We focused on simultaneous extraction of both spatial and temporal features and explored feasible methods to realize it through reinforcement learning episodes. We adopted the restricted Boltzmann machine (RBM) as a basic architecture of the learning system. In order to handle the temporal component, we introduced the recurrent connection between hidden layers in the model. The theoretical connection of the proposed statistical model with a deterministic Recurrent Neural Network (RNN) has been investigated. The ability to solve a simple partially observable Markov Decision Process (POMDP) problem was demonstrated in the simple T-maze task.

An instance-based reinforcement learning method for real-time robot control [Zhumatiy]
Piecewise-Continuous Nearest Sequence Memory (PC-NSM) algorithm is an instance-based reinforcement learning method designed to cope with multi-modal sensory inputs. To apply it to real-time robot control on the scale of observation sampling frequency (scales around 20Hz), two shortcomings were addressed: 1) the linear degradation of PC-NSM algorithm performance with growing experience size; and 2) inability of the algorithm to support infinite action sets (including continuous actions). To alleviate these deficiencies, PC-NSM's indexing data structure was replaced with a new instance indexer. The indexer is an application of recent research results in high-performance metric space indexing. It allows the PC-NSM algorithm to work with infinite (continuous) action set. The performance went up from linear in the number of instances to logarithmic, allowing the algorithm to work with experience histories 10-100 times longer than it was previously possible. To improve performance further and profit from multi-core architectures, PC-NSM implementation was parallelized and the new indexer was made thread-safe.

A feasibility study of IP core implementation of reinforcement learning [Yoshimoto, Kimura]
RL is an ideal framework for realizing automatic control and many RL methods have shown good

performance in the level of synthetic benchmarks and pure researches. However, there have been very few examples of their industrial applications yet. In order to explore the possibility of RL's contribution to industry, we tackled with an automatic preloading-control for a dynamically reconfigurable hardware (DRH), which is a IP core developed by NEC Co. Ltd. We formulated the issue as a semi-Markov process and devised an parallel temporal difference learning algorithm to predict the time of the next visit to each state. The performance of the method was demonstrated using JPEG decoder program running on a DRH simulator. The result showed that the method outperformed a widely-used "Least Recently Used (LRU)" heuristics in most cases and was comparable with the theoretically optimal algorithm whose implementation is infeasible in a realistic situation, when the size of hardware resources is much smaller than that of software resources.

3.2 Systems Neurobiology Group

Role of serotonin in actions for delayed rewards [Katsuhiko Miyazaki, Kayoko Miyazaki]

While serotonin is well known to be involved in a variety of psychiatric disorders including depression, schizophrenia, autism, and impulsivity, its role in the normal brain is far from clear despite abundant pharmacology and genetic studies. From the viewpoint of reinforcement learning, we earlier proposed that an important role of serotonin is to regulate the temporal discounting parameter that controls how far future outcome an animal should take into account in making a decision (Doya, 2002).

In order to clarify the role of serotonin in natural behaviors, we performed rat neural recording and microdialysis measurement from the dorsal raphe nucleus, the major source of serotonergic projection to the cortex and the basal ganglia.

By microdialysis experiments, we found that the level of serotonin release is significantly elevated when a delay is introduced before reward delivery in food-water navigation task. Such increase in serotonin release was not seen when the rewards were omitted in two out of every three trials. We also examined that whether manipulation of serotonergic activation influence waiting behavior for delayed reward. 5-HT1A agonist, 8-OH-DPAT was directly injected into the dorsal raphe nucleus to reduce serotonin neural activity by reverse dialysis method. We found that 8-OH-DPAT treatment increased wait error for delayed reward. These findings show the causality between activity of serotonin and effortful waiting for delayed reward.

Further, by multiple electrode recording from the dorsal raphe nucleus, we found that the serotonin neurons increased firing rated while the rat stayed at the food or water dispenser in expectation of reward delivery. The increased activity prolonged in accordance with length of delay period. When the rat abandoned to wait, it was often preceded by diminished firing of serotonin neurons. These findings also support the hypothesis that activation of serotonin is necessary for behaviors directed for delayed rewards.

Role of the striatum in a decision making [Ito]

The striatum is an input structure of the basal ganglia that contributes to the process of the decision making. Previous lesion and imaging studies suggested that the dorsal striatum (DS) contributes to action selection while the ventral striatum (VS) to reward prediction and motivation. However, it is still unclear whether and how the neuronal representations are different in VS and DS, and how VS and DS cooperate with each other in the decision making.

To explore these questions, we started to a new recording experiment of rats from FY2008. We recorded neuronal activity from the dorsolateral striatum (DLS), the dorsomedial striatum (DMS), and VS of rats performing a choice task consisting of two different types of blocks; a fixed-reward block (FRB) where stimulus-reward associations were fixed, and a varied-reward block (VRB) where the association was varied.

Currently, we focused on the neurons of which activities were changed by the feature action that rats would select (action-command; AC). Up to now, 51% (49/96), 47% (33/70) and 40% (27/68) of the neurons recorded from DLS, DMS and VS were regarded as the AC neurons, respectively (Figure 1). The average of the information of AC was significantly higher in DS than in VS (0.040, 0.037, and 0.012 bit/s for DLS, DMS, and VS). Furthermore, we found the AC neurons of which the information was higher in FRB than in VRB. These findings suggest that the action selection is more deeply involved in DS than VS, and more habituated behavior is represented more strongly in the striatum.

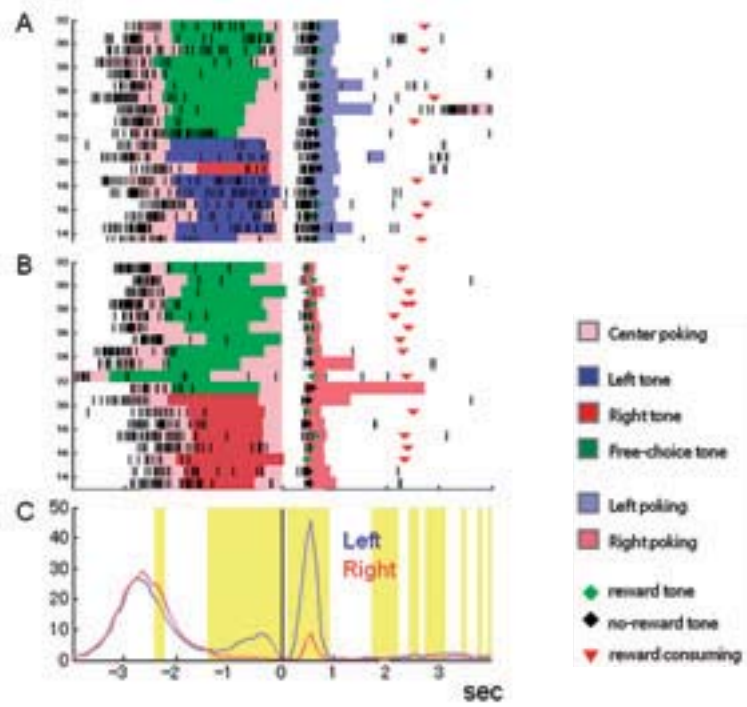

Figure 1: An example of the neurons in the dorsolateral striatum coding the action command. **A**, **B**, Each row indicates rat's behavior and neuronal spikes (black vertical lines) in one trial. The offset of the center hole poking, corresponding to the onset of the action selection, was set to 0 second. Behavioral and neuronal data of left-selected trials and right-selected trials are separately shown in A and B, respectively. The firing rate for each condition is shown in **C**. This neuron was activated before the initiation of the action when the rat would select the left poking but not when select the right poking.

Brain mechanisms for evaluating probabilistic and delayed rewards [Yoshida, Ito, Yoshimoto]

Humans prefer sooner, more probable rewards over delayed, less probable ones. To clarify such a reward-discounting mechanism, we investigated the brain activity in the presence of both probabilistic and delayed rewards using functional magnetic resonance imaging.

Ten human subjects performed a choice task in which two visual stimuli, each coding a pair of delay and probability of a juice reward, were presented in series and the subject selected one of them by pressing a button. While activity of right posterior cingulate gyrus, right superior parietal lobule and caudate correlated with the reward probability, activities of left anterior cingulate and right inferior parietal lobule correlated with the reward delay. The separate activity loci suggest distinct processing mechanisms for delay and probability discounting (Figure 2).

Activation of adjacent areas in the right parietal lobule for more probable and sooner rewards, respectively, suggests that the area integrates two sources of information on reward discounting.

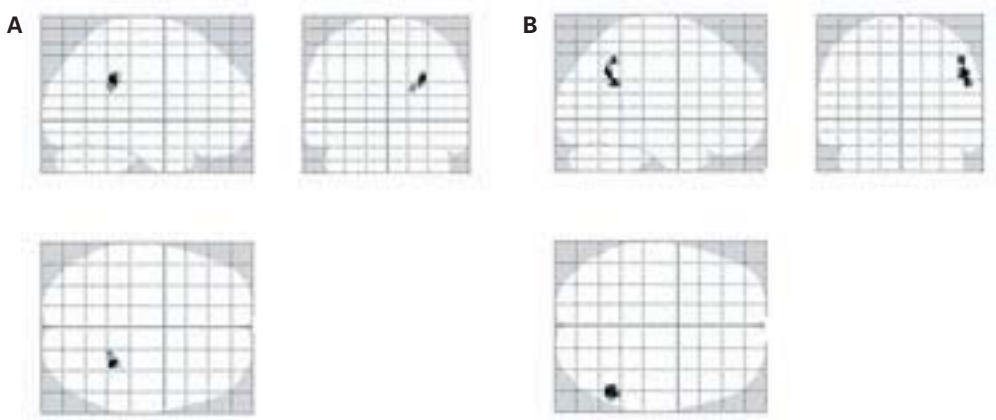

Figure 2: Brain regions related to evaluation of reward. **A**, Brain regions showing a negative correlation with the delay. **B**, Brain regions correlating with the probability.

Model-free and model-based reinforcement strategies in the acquisition and selection and of sequential actions [Fermin, Yoshida, Ito, Yoshimoto]

Reinforcement learning (RL), a computational theory of adaptive optimal control, suggests two methods for action selection that fit well to human behavior: Model-Free (MF) method uses action value functions to predict future rewards based on current states and available actions, and Model-Based (MB) method uses a forward model to predict the future states reached by hypothetical actions.

To test whether and how humans utilize MB and MF strategies, we performed an fMRI experiment using a "grid sailing task" where subjects had to move a cursor from the start to the goal position by sequentially pressing three keys assigned to the different direction that the cursor moved. The experiment was performed under three task conditions: (1) well-learned action sequences, (2) use of learned key-map for new start and goal positions, (3) use of new key-map for new start and goal positions.

During the delay period before the initiation of the action sequence, task condition 2 activated mainly anterior areas: left dorsolateral prefrontal cortex, ventral/dorsal premotor cortex, posterior cerebellum, visual cortex, right ventral premotor cortex and striatum (Figure 3). Task condition 3 recruited areas related to somatosensory and visuo-spatial information processing (Figure 3).

Areas activated in condition 2 during the delay period, within the premotor cortex and the striatum, are candidates to the network implementing MB RL method. As a future step we will perform a model-based analysis using RL algorithms on the behavioral data and seek for any tendency in the use of distinct action selection strategies as well as the neural mechanisms implementing them.

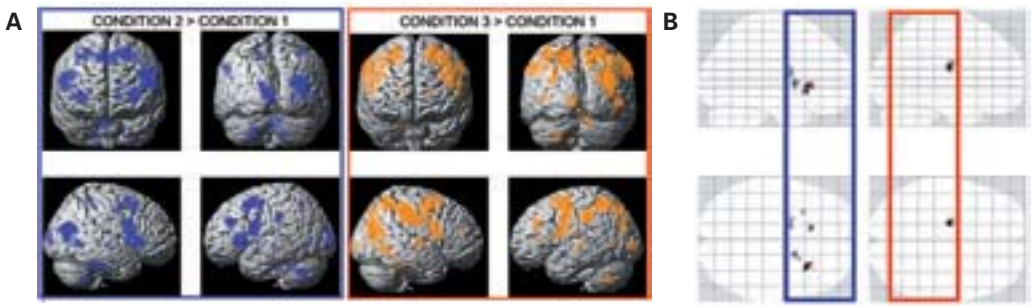

Figure 3: Brain regions related to strategies for sequential actions. **A**, Blue areas, the regions of which activities were grater in condition 2 than in condition **1**, are candidates to the network implementing model-based strategy. Orange areas show the regions of which activities were grater in condition 3 than in condition **1**. **B**, Activation of the striatum in condition 2 (blue) and condition 3 (orange).

Learning of chunking of action sequences [Hoshino]

To clarify the computational principle of chunking of action sequences and the neural mechanisms, we developed a new motor sequence task which requires the rat to learn voluntarily. Using a reward for a clue, the rat acquires the correct sequence of lever pressing on the walls of octagonal operant chamber. We used Intracranial Self-stimulation method for rewards. Surprisingly, the rats could acquire correct sequences without any instruction cues and the information of sequence length. Currently, our rats can conduct three steps motor sequence well. This learning ability is not simply based on memories of cues which experimenter showed. The rats acquire correct motor sequence with try and error. This will help to promote a chunking of action which is a kind of reusable superordinate. Hence we will investigate generation mechanism of motor chunking using this task.

3.3 Adaptive Systems Group

Finding intrinsic rewards based on the embodied evolution [Uchibe]

Understanding the design principle of reward functions is a substantial challenge both in artificial intelligence and neuroscience. Successful acquisition of a task usually requires not only rewards for goals, but also for intermediate states to promote effective exploration. This study proposes a method for designing 'intrinsic' rewards of autonomous agents by combining constrained policy gradient

Figure 4: Embodied evolution in the real environment. There exist three Cyber Rodents (CR) and many battery packs.

reinforcement learning and embodied evolution. To validate the method, we use Cyber Rodent robots (CR), in which collision avoidance, recharging from battery packs, and 'mating' by software reproduction are three major 'extrinsic' rewards (Figure 4). We found that the obtained reward function allowed CRs to acquire a variety of exploring behaviors and two correlation coefficients were computed to illustrate qualitative features of exploration. To characterize the learned behaviors, we use a running distance which indicates distance traveled by the CR. The figure shows that there existed a strong correlation between the running distance from 290 to 300 min and a parameter of the reward function (w2) averaged over the same time interval. A short running distance implied that the CR tried to find another CR or battery packs by turning clockwise or anti-clockwise. We found that the CR with small w2 sometimes oscillated between the battery pack and the robot when they were observed simultaneously. On the other hand, a long running distance showed that the CR wandered around the experimental field. The right figure also shows that there existed a weak correlation between the number of battery packs obtained from 290 to 300 min and w2.

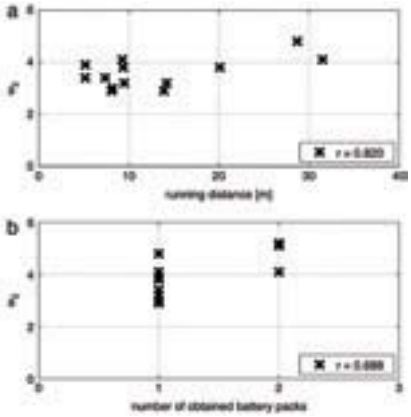

Figure 5: Differences of learned behaviors. (a) Relation between the running distance and the averaged w2 in the interval from 290 to 300 min. The running distance was calculated by summing straight-line distance between successive time steps. (b) Relation between the number of obtained battery packs and the averaged w2 in the interval from 290 to 300 min.

Co-Evolution of Rewards and Meta-Parameters in Embodied Evolution [Elfwing]

Embodied evolution is a methodology for evolutionary robotics that mimics the distributed, asynchronous, and autonomous properties of biological evolution. The evaluation, selection, and reproduction are carried out by cooperation and competition of the robots, without any need for human intervention. An embodied evolution framework is therefore well suited to study the adaptive learning mechanisms for artificial agents that share the same fundamental constraints as biological agents: self-preservation and self-reproduction. This study proposes a framework for performing embodied evolution with a limited number of robots, by utilizing time-sharing in subpopulations of virtual agents. Within this framework, we explore the combination of within-generation learning of basic survival behaviors by reinforcement learning, and evolutionary adaptations over the generations of the basic behavior selection policy, the reward functions, and meta-parameters for reinforcement learning. We apply a biologically inspired selection scheme, in which there is no explicit representation or communication of the individuals' fitness information. The individuals can only reproduce offspring by mating, a pair-wise exchange of genotypes, and the probability that an individual reproduces offspring in its own subpopulation is dependent on the individual's "health", i.e., energy level, at the mating occasion. We validate the proposed method by comparing the proposed method with evolution using standard centralized selection, in simulation, and by transferring the obtained solutions to hardware using two real robots. An interesting result is the evolution of tau, controlling the trade-off between exploration and exploitation in the softmax selection. Tau very quickly drops to approximately 0. This result means that action selection tends to be equal to the greedy action selection strategy, and always selects the action corresponding to the largest Q-value. This result indicates that in the presence of sufficiently good shaping rewards, additional exploration in the form of a stochastic policy is unnecessary and actually decreases the learning performance.

Hierarchical and Reusable Modular Representation of Artificial Neural Networks for Modular Search [Kamioka, Uchibe]

The framework of neuroevolution (NE) provides a way of finding appropriate structures as well as connection weights of artificial neural networks. However, the conventional NE approach of directly coding each connection weight by a gene is severely limited in its scalability and evolvability. In this study, we propose a novel indirect coding approach in which a phenotypical network develops from the genes encoding multiple subnetwork modules. Each gene encodes a subnetwork consisting of the input, hidden, and output nodes and connections between them. A connection can be a real weight or a pointer to another subnetwork. The structure of the network evolves by inserting new connection weights or subnetworks, merging two subnetworks as a higher-level subnetwork, or changing the

existing connections. We investigated the evolutionary process of the network structure using the pole balancing task. The following figure shows solutions obtained by evolution, where #connections and #nodes are the number of connection and the number of nodes in phenotypic neural network respectively. #sn and #weights are the size of subnetworks and weights in the genotype. There were no significant difference between the number of connections in phenotypic neural network obtained between modular and non-modular search. However, the size of weights obtained by modular search which is representing those connections was significantly less than nonmodular one. Our proposed modular search method realized compression of genetic length which is the dimension of weight parameter search space. The modular search tend to make a complex structure by small weight parameters.

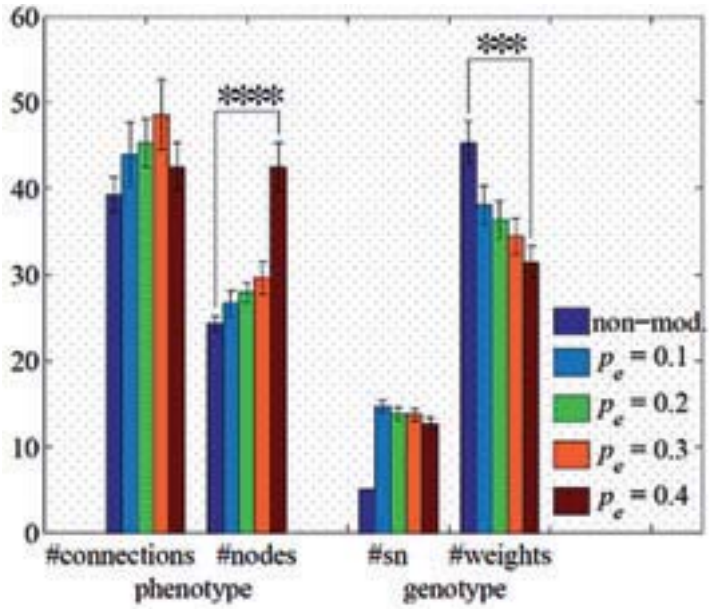

Figure 6: Simulation results of the parallel pole-balancing without velocity information (PPVN) task (∗: P < 0.1, ∗∗: P < 0.001, ∗∗∗: P < 0.0005, ∗∗∗∗: P < 0.00005). The modular search could find a solution faster than the non-modular search.

Vision based navigation by reinforcement learning and visual servoing [Kobayashi, Uchibe]

The learning speed of reinforcement learning is too slow to be useful when it is applied to real-world problems. One reason is that the state space grows exponentially with the number of sensory inputs such as image measurements, joint angles when we apply reinforcement learning simply. The goal of this research is to reduce the dimension of the state spaces in the framework of reinforcement learning. The basic idea is to introduce a feedback controller into a part of the robotic system and extract state variables based on the feedback control. Our proposed system consists of two parts: adaptive visual servoing and reinforcement learning. The adaptive visual servoing minimizes the distance between the current and desired image measurements by controlling joint angles of the camera-head mounted on the robot. If the position of the target object remains unchanged, the final joint angles can be regarded as the position of the robot in the environment. On the other hand, the role of reinforcement learning is to drive the robot to the target object in the environment. The state space is constructed from the converged joint angles of the camera head. To verify our proposed method, we make experiments by using a legged robot called AIBO. Experimental results show that the converged joint angles represent the position of the robot in the environment appropriately.

Learning locomotive behaviors for quadruped robots [Haga, Uchibe]

The goal of this study is to develop a robust learning framework for a new quadruped robot with passive knee joints. As compared with the mobile robot such as Cyber Rodent, it is very difficult to realize stable locomotive behaviors due to its non-linear dynamics. As the first step, we investigated the dynamics by applying a simple feedforward controller and measured accelerometers during running.

4 Publications

4.1 Journals

Bissmarck, F., Nakahara, H., Doya, K., Hikosaka, O. Combining modalities with different latencies for optimal motor control. *Journal of Cognitive Neuroscience* 20,1966-1979 (2008).

Doya, K. Modulators of decision making. *Nature Neuroscience* 11, 410-416 (2008).

Doya, K. Computers in the brain: Network and molecular mechanisms in cerebellum, basal ganglia, hippocampus, and cerebral cortex (in Japanese). *Seitai-no Kagaku* 59, 10-19 (2008).

Doya, K. What is the objective function of the brain (in Japanese). *Instrumentation and Control* 47 (1), 69-73 (2008).

Elfwing, S., Uchibe, E., Doya, K., Christensen, H.I. Co-evolution of shaping rewards and meta-parameters in reinforcement learning. *Adaptive Behavior* 16 (6), 400-412 (2008).

Elfwing, S., Uchibe, E., Doya, K., Christensen, H.I. Darwinian Embodied Evolution of the Learning Ability for Survival. *Adaptive Behavior* (in press).

Fujiwara, Y., Yamashita, O., Kawawaki, D., Doya, K., Kawato, M., Toyama, K., Sato, M. A hierarchical Bayesian method to resolve an inverse problem of MEG contaminated with eye movement artifacts. *Neuroimage* 45(2), 393-409 (2008).

Ito, M., Doya, K. Mathematical models of decision making and learning (in Japanese). *Brain and Nerve* 60 (7), 791-798 (2008).

Ito, M., Shirao, T., Doya, K., Sekino, Y. Three-demensional distribution of Fos-positive neurons in the supramammillary nucleus induced by exploring behavior of rats. *Neuroscience research* (in press).

Morimura, T., Uchibe, E., Yoshimoto, J., Doya, K. A new natural gradient of average reward for policy search (in Japanese). *The Institute of Electronics, Information and Communication Engineers* J91-D (6), 1515-1527 (2008).

Morimura, T., Uchibe, E., Yoshimoto, J., Peters, J., Doya, K. Derivatives of logarithmic stationary distributions for policy gradient reinforcement learning. *Neural Computation* (in press).

Otsuka, M., Yoshimoto, J., Doya, K. Reward-dependent sensory coding in free-energy-based reinforcement learning. *Neural Network World*. (in press).

Sato, H., Uchibe, E., Doya, K. Emergence of cooperation and communication in reinforcement learning agents. *Proceedings of Information Processing Society of Japan: Mathematical Modeling and Application* 48 (TOM19), 55-67 (2008)

Schweighofer, N., Bertin, M., Shishida, K., Okamoto, Y., Tanaka, S., Yamawaki, S., & Doya, K. Low-serotonin levels increase delayed reward discounting in humans. *Journal of Neuroscience* 28, 4528-4532 (Erratum in 4528, 5619). (2008).

Uchibe, E., Doya, K. Finding intrinsic rewards by embodied evolution and constrained reinforcement learning. *Neural Networks* 21:1447-1455 (2008).

4.2 Book(s) and other one-time publications

Doya, K. Neural network and molecular mechanisms of reinforcement learning (in Japanese). Brain Science Foundation (ed) Brain Science Review 2009. Kuba Pro (2009)

Doya, K., Kimura, M. The basal ganglia and the encoding of value. In Glimcher, P. W., Camerer, C. F., Fehr, E., Poldrack, R. A. (eds.) Neuroeconomics: Decision Making and the Brain, 407-416. Elsevier

(2009)

Elfwing, S., Uchibe, E., Doya, K. Co-evolution of rewards and meta-parameters in embodied evolution. In Sendhoff B, Koerner E, Sportns O, Ritter H, Doya K (eds.) Creating Brain-like Intelligence, 278-302. Springer-Verlag (2009)

Sendhoff, B., Koerner, E., Sportns, O., Ritter, H., Doya, K. Creating Brain-like Intelligence. Springer-Verlag (2009)

4.3 Oral presentations

Doya, K. Neural mechanisms of cognitive and motor control in the basal ganglia and the cerebellum (in Japanese). Kyoto Advanced Occupational Therapy Seminar. Kyoto, April 5, 2008.

Doya, K. Solving differential equations by MATLAB. Okinawa Computational Neuroscience Course. Onna, June 15-July 4, 2008.

Doya, K. Reinforcement learning and the basal ganglia (in Japanese). Japanese Basal Ganglia Society. Fuji, July 5-6, 2008

Doya, K. Regulation of patience by serotonin. International Symposium on Attention and Performance XXII. Bermont, USA, July 14-17, 2008

Doya, K. Neural circuit and molecular mechanisms of reinforcement learning (in Japanese). Biochemistry Summer School. Tokyo, August 1-3, 2008

Doya, K. Brain circuit and molecular mechanisms of reinforcement learning (in Japanese). Physiology Summer School, Tokyo, August 4, 2008

Doya, K. Neuroscience and decision making: questions answered and questions emerging. Workshop on Open Problems in Neuroscience of Decision Making, Onna, October 16-18, 2008

Doya, K. Understanding the brain by creating it (in Japanese). OIST Open House, Uruma, November 9, 2008

Doya, K. Computation and neural mechanism for reinforcement learning. Plenary Lecture, International Conference on Neural Information Processing. Auckland, November 25-28, 2008

Doya, K. Brain mechanisms of reinforcement learning (in Japanese). Basic Integrative Lecture, University of Tokyo Medical School. Tokyo, December 15, 2008

Doya, K. Thinking of life, intelligence, and mind through robots (in Japanese). Science Talk and Film Show: Near Future Robots. Naha, February 15, 2009

Doya, K. Model-free and model-based strategies in motor sequence learning and control. Dynamic Brain Forum 2009. Atami, March 2-4, 2009

Doya, K. Computational theory of action learning and plastic mechanisms of the cell (in Japanese). Senri Life Science Seminar: Computing Mechanisms of the Cell: From ES Cells to Neurons. Senri, March 16, 2009

Fermin, A., Yoshida, T., Tanaka, S., Ito, M., Yoshimoto, J., Doya, K. Model-Free and Model-Based Reinforcement Learning Strategies in the Acquisition and Selection of Sequential Actions. The 19th Annual Conference of Society for the Neural Control of Movement (NCM), Hawaii, US. To appear in April 28-May 3, 2009

Kamioka, T., Uchibe, E., Doya, K. NeuroEvolution based on Reusable and Hierarchical Modular Representation. INNS-NNN Symposia (New directions in Neural Networks) 2008, Auckland, New

Zealand,November 24-25, 2008

Morimura, T., Uchibe, E., Yoshimoto, J., Doya, K. A new natural policy gradients by stationary distribution metric. The European Conference on Machine Learning and Principles and Practice of Knowledge Discovery in Databases (ECML-PKDD2008), Antwerp, Belgium, September 15-19, 2008

Nakano, T. Theoretical and experimental study of synaptic plasticity in striatum. Australian Course in Advanced Neuroscience (ACAN). Australia, April 20-May 10, 2008

Otsuka, M., Yoshimoto, J., Doya, K. Robust population coding for a free energy based reinforcement learning agent. The 18th International Conference on Artificial Neural Networks (ICANN 2008), Prague, Czech Republic, September 3-6, 2008

Otsuka, M., Yoshimoto, J., Doya, K. Task-dependent distributed representation of latent states in free-energy-based reinforcement learning. Regular Meeting of Neurocomputing Technical Group in The Institute of Electronics, Information and Communication Engineers, Okinawa, Japan, June 26-27, 2008

Uchibe, E. Learning and Evolution in Real Robots. The 3rd International CINACS Summer School 2008. Hamburg, Germany, September 8-12, 2008

Uchibe, E. Reinforcement Learning from intrinsic and extrinsic rewards. The 11th Workshop on Information-Based Induction Sciences (IBIS 2008), Sendai, Japan, October 29-31, 2008

Yoshida, T., Ito, M., Morimura, T., Samejima, K., Okuda, J., Yoshimoto, J., Doya, K. A study of decision-making for delayed and probabilistic delay. The 13th Regular Meeting of Special Interest Groups of Bioinformatics and Genomics, Okinawa, Japan, June 26-27, 2008

Yoshimoto, J., Nakano, T. Machine learning v.s. learning systems in brain: Fusion of learning theory and systems biology. The 3rd. Workshop for young researchers in System Cell Engineering by Multi-scale Manipulation, Naha, Japan, July 11, 2008

4.4 Posters

Fermin, A., Yoshida, T., Yoshimoto, J., Doya, K. Role of internal models in planning and learning of sequential behaviors. The 9th Summer Workshop on Mechanism of Brain and Mind. Sapporo, Japan, August 9-10, 2008

Ito, M., Doya, K. The dorsal-ventral and anterior-posterior functional difference within the striatum in goal-directed and habitual behavior. The 9th Summer Workshop on Mechanism of Brain and Mind. Sapporo, Japan, August 9-10, 2008

Nakano, T., Yoshimoto, J., Wickens, J., Doya, K. Timing-dependent calcium response model in striatal spiny neurons. The 9th Winter Workshop on Mechanism of Brain and Mind. Rusutsu, Japan, January 13-15, 2009

Vickers, C., Nakano, T., Wickens, J. Dopamine dependent plasticity in the corticostriatal system. The 6th Forum of european neuroscience. Geneva, Switzerland, July 12-16, 2008

Yoshimoto, J. Bayesian system identification for intracellular signalling cascades. The 5th Japanese-German Frontiers of Science Symposium (JGFoS). Mainz, Germany, October 30-November 2, 2008

Yoshimoto, J., Inoue, T., Doya, K. Development of GUI software for Bayesian signal transduction identification. The 9th Winter Workshop on Mechanism of Brain and Mind. Rusutsu, Japan, January 13-15, 2009

5 Intellectual Property Rights and Other Specific Achievements

Nothing to report.

6 Meetings and Events

6.1 Seminar

Date: June 30, 2008
Venue: OIST IRP Conference Room
Speaker: Satoshi Kojima (U California, San Francisco)

6.2 Summer Workshop on the Mechanisms of Brain and Mind

Date: August 9-10, 2008
Venue: Sapporo
Co-sponsor: Integrative Brain Research Project
Speakers: Matthew Roesch (U Maryland)
Okihide Hikosaka (NIH)
John O'Doherty (U Dublin)

6.3 Workshop on Open Problems in Neuroscience of Decision Making

Date: October 16-18, 2008
Venue: OIST Seaside House
Co-organizers: Bernard Balleine (UCLA)
Hiroyuki Nakahara (RIKEN)
John O'Doherty (U Dublin and Caltech)
Masamichi Sakagami (Tamagawa U)
Co-sponsors: RIKEN Brain Science Institute
Tamagawa University
University of California, Los Angeles
California Insitute of Technology
Speakers: Bernard Balleine (UCLA)
Thomas Boraud (CNRS)
Peter Bossaerts (EPFL)
Paul Cisek (U Montreal)
Nathaniel Daw (New York U)
Kenji Doya (OIST)
Michael Frank (U Arizona)
Jon Horvitz (Boston College)
Brian Hyland (U Otago)
Yasushi Kobayashi (Osaka U)
Hiroyuki Nakahara (RIKEN)
Yael Niv (Princeton U)
John O'Doherty (U Dublin and Caltech)
Antonio Rangel (Caltech)
Masamichi Sakagami (Tamagawa U)
Geoffrey Schoenbaum (U Maryland)
Mark Walton (U Oxford)

6.4 Winter Workshop on the Mechanisms of Brain and Mind

Date: January 13-15, 2009
Venue: Rusutsu Resort
Co-sponsor: Integrative Brain Research Project
Speakers: James Kozloski (IBM)
Sebastian Seung (MIT)
Marcel Oberlaender (Max-Planck-Institute)
Erik De Schutter (OIST)
Toshihiko Hosoya (RIKEN)

Reiko Kobayakawa (JST PRESTO)
Ryohei Kanzaki (U Tokyo)
Hiroaki Gomi (NTT)
Kazuhiko Seki (NIPS)

6.5 Seminar

Date: January 16, 2009
Venue: OIST IRP Conference Room
Speaker: Marcel Oberlaender (Max Planck Institute)

6.6 Seminar

Date: February 12, 2009
Venue: OIST IRP Conference Room
Speaker: Yusuke Tsuno (U Tokyo)

6.7 Seminar

Date: February 20, 2009
Venue: OIST IRP Conference Room
Speaker: Shiro Ikeda (Institute of Statistical Mathematics)

6.8 Workshop on Mathematical Modeling and Problem Solving

Date: March 5-6, 2009
Venue: OIST Seaside House
Co-sponsor: Information Processing Society of Japan
Speakers: 58 domestic speakers
http://daemon.ice.uec.ac.jp/MPSPortal/events/7b2c7356demps78147a764f1a
Other remarks: Co-sponsored Workshop

6.9 Neural and Brain Systems Team Meeting

Date: March 8-9, 2009
Venue:OIST Seaside House
Co-sponsor: Computational Science Research Program
Speakers: Yasuhiro Sunaga (RIKEN)
Akira Amano (Kyoto U)
Yosuke Ohno (RIKEN)
Shin Ishii (Kyoto U)
Shinya Kuroda (U Tokyo)
Markus Diesmann (RIKEN)
Tomoki Fukai (RIKEN)
Ryohei Kanzaki (U Tokyo)
Shiro Usui (RIKEN)
Kenji Doya (OIST)
Tadashi Ogawa (Kyoto U)
Yasushi Kobayashi (Osaka U)
Other remarks: Co-sponsored Workshop

Unit for Molecular Neurobiology of Learning & Memory

Principal Investigator:
Shogo Endo

Research Theme:
Molecular and genetic dissection of learning and memory

Abstract

The brain, the organ of memory and cognition, is supported by the result of the fine-tuned molecular mechanisms in neuronal cells. The functions of neuronal cells are the origin for all mental activities including memory. One of the fundamental questions in the field of neuroscience is to understand the molecular mechanisms underlying memory. Memory provides basis for the other higher brain function such as thought, language, and emotion. We have studied the molecular and cellular aspect of memory based on the techniques of biochemistry, molecular biology and gene targeting. We continue to attack the biochemical and cellular aspects of the neuronal plasticity and memory as models of brain functions to reveal intimate machinery of the brain.

1 Staff

Researchers: Gilyana Borlikova, Ph.D.
Jayne Nicole Rafferty, Ph.D.
Toshiro Sakamoto, Ph.D.
Réjan Vigot, Ph.D.
Technical Staff: Masako Suzuki, M.Sc. (Laboratory Manager)
Michiko Arai, B.Sc.
Tomoko Arasaki, M.Sc.
Ms Nana Noumi
Mika Takiguchi, M.Sc.
Graduate Student: Yukiko Uechi, M.Sc.
Research Administrator / Secretary: Ms Shoko Yamakawa

2 Partner Organizations

RIKEN Brain Science Institute
Type of partnership: Collaborative Research
Name of principal researcher: Dr. Masao Ito
Research theme: Electrophysiological examination of genetically modified mice.

RIKEN Brain Science Institute
Type of partnership: Collaborative Research
Name of principal researcher: Dr. Soichi Nagao
Research theme: Behavioral examination of genetically modified mice.

Shinshu University Graduate School of Medicine
Type of partnership: Collaborative Research

Name of principal researcher: Dr. Tatsuo Suzuki
Name of researcher: Dr. Q. B. Tian
Research theme: Comprehensive characterization of mRNAs localized in synapse.

National Defense Medical College
Type of partnership: Collaborative Research
Name of principal researcher: Prof. Kunio Takishima
Name of researcher: Dr. Yasushi Satoh
Research theme: Generation of the mice with modified ERK2 gene.

3 Activities and Findings

We are dissecting signal transduction cascade involved in neuronal plasticity and memory in two categories; early phase and long-lasting phase (late phase): The latter requires newly synthesized proteins created through gene transcription and translation. We attack the molecular mechanism underlying neuronal plasticity and learning and memory using a variety of methods including biochemistry, molecular biology, behavioral analysis and also gene manipulation technology.
Memory can be observed only by the examining the behaviors of animals. Even though the neuronal plasticity is believed to be the cellular basis of the memory, the memory itself requires the neuronal network based on the neuronal cell interaction, the brain and the whole body. We chose the mice as a model animal for the study on memory. The gene manipulation of mice is well established and it is possible to obtain gene-deficient mice and transgenic mice in our lab from scratch. We utilized the genetically-modified mice to examine the molecular mechanisms underlying learning and memory.

3.1 G-substrate and motor learning (Endo et al., 2009)

G-substrate was identified as a substrate of PKG by Paul Greengard's group in the early 1980s. Since then, efforts to clone G-substrate cDNA have failed, despite numerous efforts. We successfully cloned rat, mouse, and human G-substrate cDNA and have generated a variety of analysis tools e.g. cDNA probes, antibodies, and G-substrate mutant mice (Endo et al, 1999). We have also identified the protein phosphatase inhibitory activity of G-substrate upon phosphorylation of the protein by PKG (Endo et al., 1999; 2003). The shuttling of G-substrate between the nucleus and cytosol in Purkinje cells may also suggest roles of G-substrate in both compartments (Suzuki and Endo, unpublished).
G-substrate is localized in restricted cells within the retina (Nakazawa et al., 2007) and substantia nigra (Chung et al., 2007) as well as its high expression in cerebellar Purkinje cells. In both retinal amacrine cells and substantia nigra A10 cells, G-substrate plays a role in neuroprotection against NMDA-induced injury (Ca^{2+}-induced injury). The retina project is being carried out in collaboration with Dr. Nakazawa in the Department of Ophthalmology, Tohoku University Medical School; and the substantia nigra project is being carried out in collaboration with Dr. Isacson at Harvard Medical School.
Further we have generated the mice deficient in G-substrate gene and explored their specific functional deficits. General behaviors of G-substrate KO mice were apparently normal and they reproduce normally. G-substrate-deficient Purkinje cells in slices obtained at postnatal week (PW) 10-15 maintained electrophysiological properties essentially similar to those from wild-type littermates. Conjunction of parallel fiber stimulation and depolarizing pulses induced long-term depression (LTD) normally. At younger ages, however, LTD attenuated temporarily at PW6 and recovered thereafter (Figure 1).
In parallel with LTD, short-term (1 hr) adaptation of optokinetic response (OKR) temporarily diminished at PW6 (Figure 2). Young adult G-substrate knockout mice tested at PW12 exhibited no significant differences from their wild-type littermates in terms of brain structure, general behavior, locomotor behavior on rotor rod or treadmill, eyeblink conditioning, dynamic characteristics of OKR, or short-term OKR adaptation. One unique change detected was a modest but significant attenuation in the long-term (5 days) adaptation of OKR (Figure 3). The present results support the concept that LTD is causal to short-term adaptation, and reveal the dual functional involvement of G-substrate in neuronal mechanisms of the cerebellum for both short-term and long-term adaptation. Recently, it is reported that the engram of short-term OKR adaptation is in cerebellar cortex and long-term one is in vestibular nuclei. The G-substrate KO mice may have molecular deficit in the conversion of short-term memory to long-term memory. The mouse line is a great model to reveal the molecular mechanisms underlying long-term memory (Endo et al., 2009).

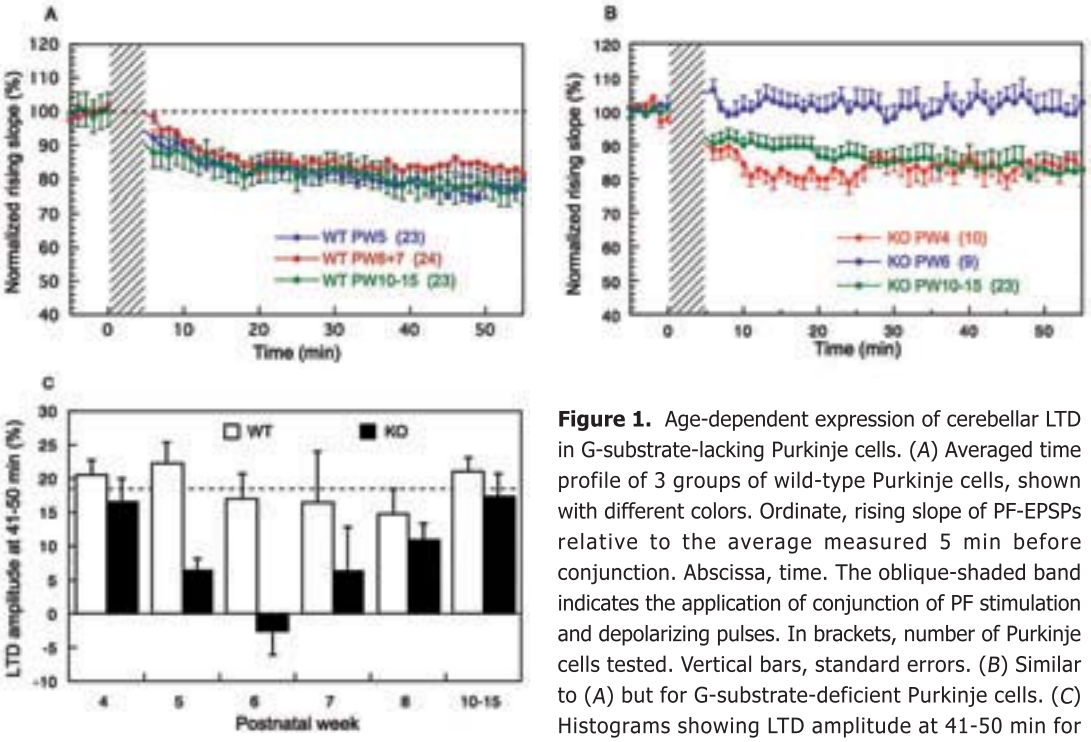

Figure 1. Age-dependent expression of cerebellar LTD in G-substrate-lacking Purkinje cells. (*A*) Averaged time profile of 3 groups of wild-type Purkinje cells, shown with different colors. Ordinate, rising slope of PF-EPSPs relative to the average measured 5 min before conjunction. Abscissa, time. The oblique-shaded band indicates the application of conjunction of PF stimulation and depolarizing pulses. In brackets, number of Purkinje cells tested. Vertical bars, standard errors. (*B*) Similar to (*A*) but for G-substrate-deficient Purkinje cells. (*C*) Histograms showing LTD amplitude at 41-50 min for different PWs in wild-type and G-substrate-deficient Purkinje cells. Filled columns are G-substrate knockout mice, and empty columns are wild-type littermates. Vertical bars, standard errors. The numbers of cells used for the experiments are as follows; for wild-type mice PW4 (12), PW5 (23), PW6 (14), PW7 (10), PW8 (14), PW10-15 (23); for G-substrate knockout mice PW4 (10), PW5 (9), PW6 (9), PW7 (6), PW8 (11), PW10-15 (29).

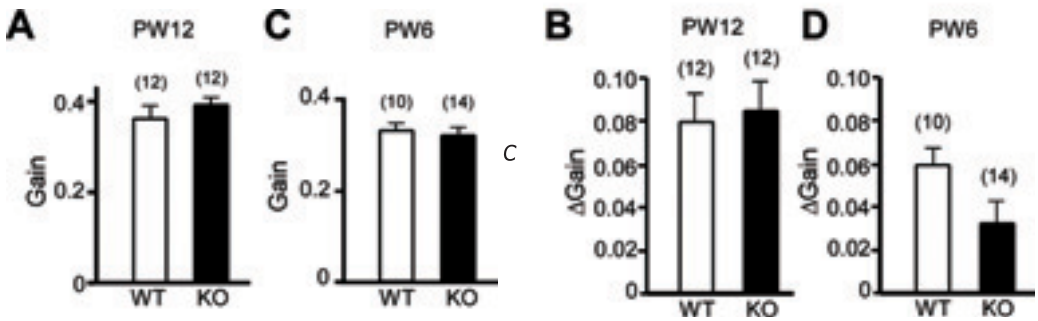

Figure 2. OKR-gain and short-term OKR-adaptation. Mice were subjected to sinusoidal screen oscillation by 15° (peak-to-peak) at 0.17 Hz (maximum velocity, 7.9 °/sec) in the light for 1 hr. (*A*) Start OKR gain before 1-hr screen oscillation at PW12. (*B*) OKR gain change obtained during 1-hr sustained screen oscillation at PW12. (*C*) Similar to A but at PW6. (*D*) Similar to B but at PW6. Filled columns, G-substrate knockout mice. Empty columns, wild-type mice. Vertical bars, standard errors. The number of mice used is shown in brackets.

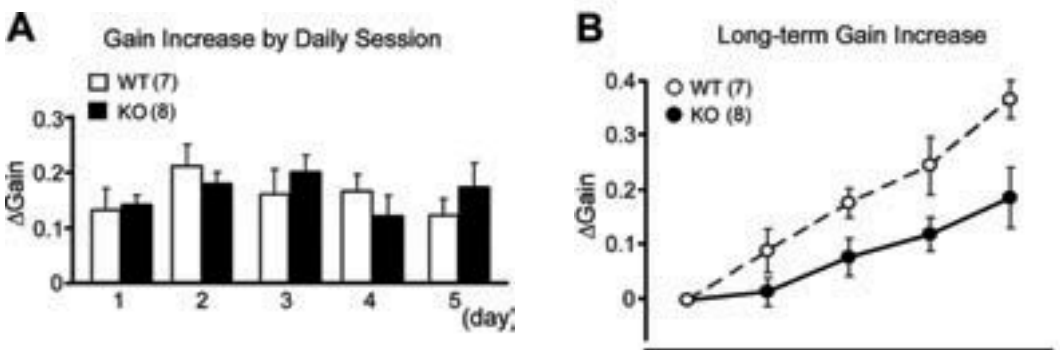

Figure 3. Long-term adaptation of OKR. Wild-type (n=7) and G-substrate knockout mice (n=8) at PW12-15 were exposed to 1-hr sustained screen oscillation every day for 5 days. The mice were kept in the dark except during the screen oscillation. (*A*) Daily OKR adaptation. Ordinate, ΔGain, gain changes obtained after 1-hr

oscillation on each day. Filled columns are G-substrate knockout mice, and empty columns are wild-type littermates. (*B*) Cumulative OKR gain changes induced through 5-day sessions. Ordinate, ΔGain, increases of the start gains on each day as measured from the start gain at day 1. Hollow circles are for wild-type, and solid circles for G-substrate knockout mice.

3.2 Cerebellum-dependent learning (delay eyeblink conditioning; Sakamoto and Endo, 2008)

Recent progress of genetically modified mice gives us the opportunity to reveal molecular mechanisms for eyeblink conditioning. Eyeblink conditioning in rabbits is well established and detailed studies have been conducted, however basic knowledge on eyeblink conditioning in mice is limited. This fact promotes us to conduct detailed research on eyeblink conditioning in mice. The role of γ-aminobutyric acid$_A$ (GABA$_A$) receptors in the deep cerebellar nuclei (DCN) was examined for delay eyeblink conditioning and rotor rod tests in mice. Bilateral injection of GABA$_A$ receptors agonist muscimol (MSC) and antagonist picrotoxin (PTX) induced significant impairment of motor coordination in rotor rod test, but the performance was recovered within 24 h after the injection (Figure 4). In the acquisition test, the learned eyeblink responses (LER) were significantly impaired by bilateral injections of MSC and PTX (Figure 5). However, PTX –injected mice might learn acquired the task latently. In addition, MSC or PTX impaired the retention of the acquired LER in the 7-days retention tests (Figure 6). Furthermore, the acquired LER were impaired by ipsilateral injection of MSC and PTX. Contralateral MSC-injection also impaired the acquisition of LER, but contralateral PTX-injection did not impaired eyeblink conditioning completely. These results revealed that GABA$_A$ receptors in the DCN play essential roles in mouse eyeblink conditioning.

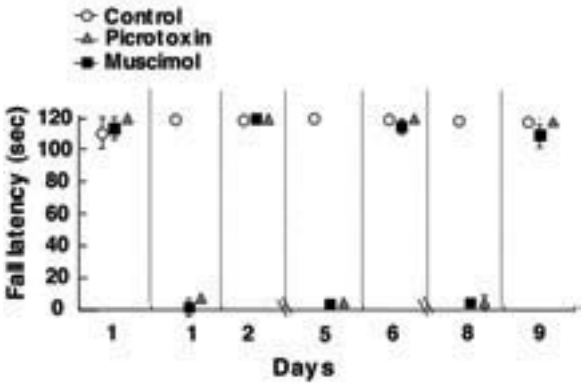

Figure 4. Averaged fall latencies in the rotor rod tests. Five trials were conducted on day 1, and average fall latencies for the last 3 trials were obtained. The retention tests were conducted 15 min after drug injections on day 1, day 5, and day 8, and the recovery tests 24h after drug injections were conducted before drug injection on day 2, day 6, day 9. Error bars indicate standard error of means (SEM). Averaged fall latencies for the mice received in muscimol (MSC: *n* = 7) or picrotoxin (PTX: *n* = 5). The control group consisted of mice injected with aCSF (*n* = 4) or without drug injection (*n* = 3). These two control groups were combined because there was no difference in performances between two control groups. Daily injections of MSC, PTX, or aCSF were carried out for 8 days.

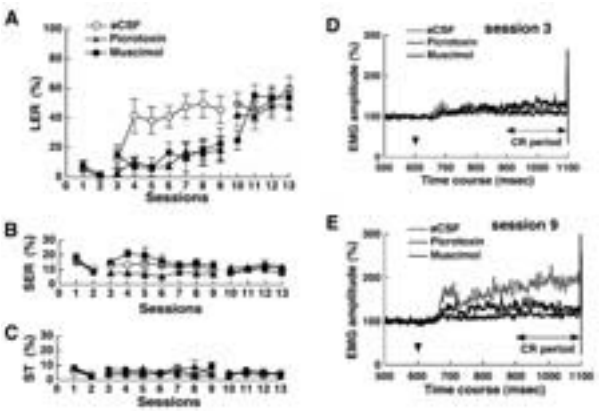

Figure 5 The acquisition test of eyeblink conditioning.
A, B, C: Learned eyeblink responses (LER) % (A), spontaneous eyeblink responses (SER) % (B), Startle eyeblink responses (STR) % (C) are shown in muscimol (MSC: *n* = 10), picrotoxin (PTX: *n* = 10), and aCSF-injected (*n* = 9) mice. Basal eyeblink responses were recorded without tone and shock in session 1-2. Each group of mice received eyeblink conditioning in session 3-13. MSC, PTX, or aCSF was bilaterally injected in sessions 2-9, and aCSF was injected in all groups in the session 10-13. Error bars indicate SEM. LER% was obtained by subtracting SER% from CR% (LER% = CR% - SER%). Error bars indicate SEM. **D, E:** The normalized EMG amplitudes in session 3 (**B**) and session 9 (**C**). EMG amplitudes of the valid trials were normalized by the mean EMG for 300 ms before the CS onset in each mouse and averaged in each group. An arrow indicates the onset of CS.

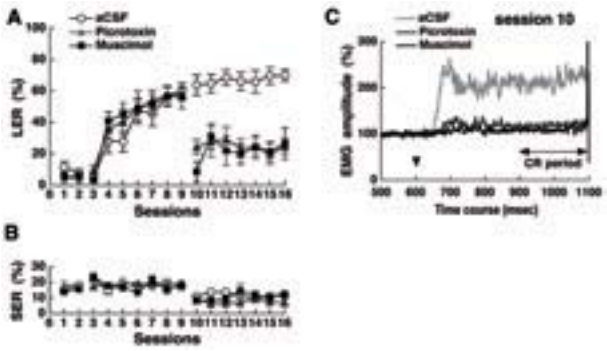

Figure 6. The retention test of eyeblink conditioning.
A, B: Learned responses (LER) % (**A**) and spontaneous eyeblink responses (SER) % (**B**) are shown the mice received in muscimol (MSC: *n* = 9), picrotoxin (PTX: *n* = 11), or aCSF (*n* = 9). Basal eyeblink responses were recorded without the tone and shock in session 1-2. Each group's mice received eyeblink conditioning without drug injection in session 3-9, and with the injection of MSC, PTX, or aCSF in session 10-16. Error bars indicate SEM. C: The normalized EMG amplitudes in session 10.

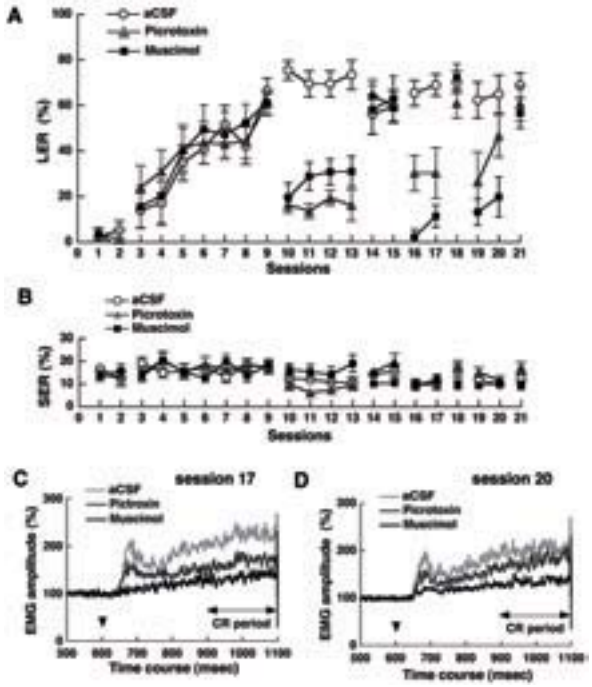

Figure 7. The laterality test of eyeblink conditioning. **A, B:** Learned responses (LER) % (**A**), and spontaneous eyeblink responses (SER) % (**B**) are shown the mice received muscimol (MSC: *n* = 9), picrotoxin (PTX: *n* = 9), or aCSF (*n* = 9). Basal eyeblink responses were recorded without tone and shock in session 1-2. All mice received eyeblink conditioning without the drug injection in session 3-9. Then, each group of mice received MSC, PTX, or aCSF bilaterally (session 10-13), ipsilaterally (session 16-17), and contralaterally (session 19-20). In the recovery sessions (session 14, 15, 18, 21), all groups of mice received eyeblink conditioning without drug injections. Error bars indicate SEM. Error bars indicate SEM **B, C:** The normalized EMG amplitudes at the ipsilateral injection session (**B**, session 17), and contralateral injection session (**C**, session 20).

3.3 Inducible cAMP early repressor (ICER)

Long-term memory (Kojima et al., 2008; Borlikova and Endo, 2009)

Long-lasting neuronal plasticity as well as long–term memory (LTM) is an event that requires new protein synthesis through nuclear transcriptional activation. The importance of gene regulation through CRE-mediated transcription is well established for LTM in a variety of animals including *Aplysia, Drosophila, C. elegans, and vertebrates*. A strong connection between CREB and neuronal plasticity underlying learning and memory has been demonstrated; in mammals, the outcome of CRE-mediated gene transcription depends on the competitive binding of CREB and CREB competitors. These factors are products of the CREB/CRE modulator (CREM)/activating transcription factor 1 (ATF-1) gene family. Despite the accumulated evidence, the role of CREM-related inducible genes, such as ICER, on neuronal plasticity and memory consolidation remains unknown.

Although basal ICER levels in the brain are relatively low, drastic upregulation of ICER has been demonstrated in responses such as neuronal excitation, suggesting that ICER may have a role in the regulation of higher-order brain functions. We observed that ICER levels in the brain increased transiently after kindled seizures and after fear conditioning.

To examine the role of ICER in LTM and kindling, we generated two types of ICER mutant mice: ICER-overexpressing (OE) and ICER knockout (KO) mice. Both ICER-OE and ICER-KO mice show no apparent abnormalities in their development and reproduction. A comprehensive battery of behavioral tests revealed no robust changes in locomotor activity, sensory and motor functions, and emotional responses in the mutant mice. However, long-term conditioned fear memory was attenuated in ICER-OE mice and enhanced in ICER-KO mice without concurrent changes in short-term fear memory (Figure 8 and 9). Furthermore, ICER-OE mice exhibited retardation of kindling

development, while ICER-KO mice, conversely, exhibited acceleration of kindling (Figure 10). These results strongly suggest that ICER negatively regulates the neuronal processes required for long-term fear memory and neuronal plasticity underlying kindling epileptogenesis, possibly through suppression of CRE-mediated gene transcription (Figure 11).

We further examined the electrophysiological aspects of ICER mutant mice. In the long-term potentiation (LTP) in hippocampus, the induction of LTP by theta-bursting stimuli was very similar among wild type, ICER-KO and ICER-OE in their amplitude and time course. However, ICER-KO mice had a tendency to lose potentiation quicker than WT and ICER-OE mice whose potentiation remained 4 hours after the induction of LTP. We will further carry out the electrophysiological characterization of these lines of mice to reveal the physiological functions of ICER in the brain.

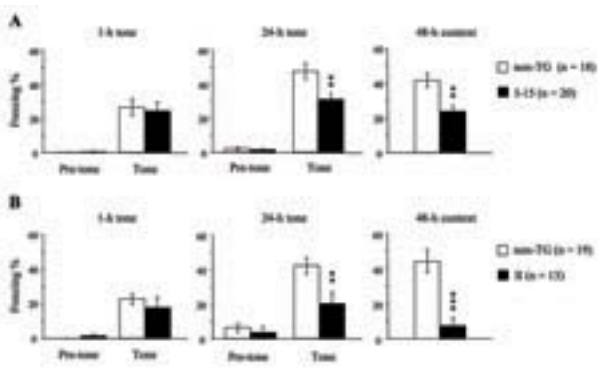

Figure 8. Fear conditioning in ICER-OE mice. Conditioned freezing to tone and context was measured in lines I-15 (***A***) and II (***B***) and was compared to conditioned freezing displayed by non-transgenic littermates (non-TG). No difference was observed between non-TG mice and both ICER-OE lines of mice at 1 h after conditioning (1-h tone). However, 24 h (24-h tone) and 48 h (48-h context) after conditioning, tone-dependent and context-dependent freezing, respectively, were significantly attenuated in ICER-OE mice. Data are means ± SEM; number of animals tested are in parenthesis. Compared to non-TG littermates: ** $p < 0.01$, *** $p < 0.001$.

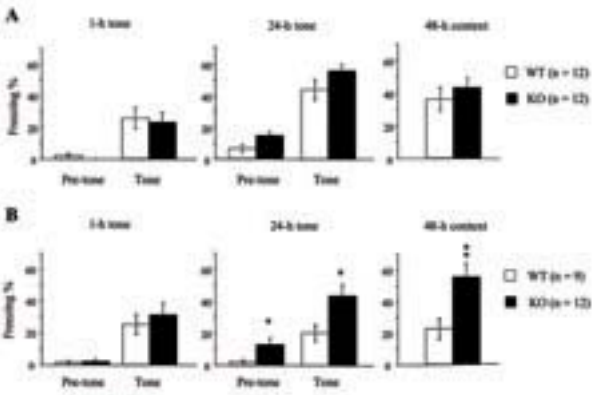

Figure 9. Fear conditioning in ICER-KO mice. ***A***, Conditioned freezing to tone and context after subjecting mice to a strong training protocol. KO mice and WT littermates exhibited similar freezing levels during the tone-dependent test performed 1 h (1-h tone) and 24 h (24-h tone) after conditioning and during the context-dependent test performed 48 h after conditioning (48-h context). ***B***, Conditioned freezing to tone and context after subjecting mice to a weak training protocol. We observed no significant differences in conditioned freezing between KO and WT mice 1 h after conditioning (1-h tone). However, KO mice showed increased freezing during both pre-tone and tone presentation at the tone-dependent test performed 24 h after conditioning (24-h tone). Context-dependent freezing in KO mice was also enhanced when tested 48 h after conditioning (48-h context). Data are means ± SEM; number of animals are in parenthesis. Compared to WT littermates: * $p < 0.05$, ** $p < 0.01$.

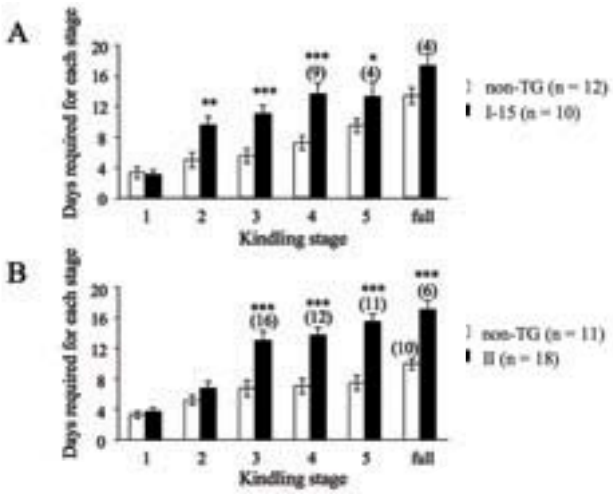

Figure 10. Amygdala kindling in ICER-OE and ICER-KO mice. Days required to reach each convulsive stage. Mice were defined as fully kindled ("full" in figure) when they had stage 5 or severer convulsions on three consecutive days: ICER-OE mice I-15 (***A***), II (***B***), and ICER-KO mice (***C***). Data are means ± SEM. Numbers in parenthesis: (n=x), total mice number per group; (y), number of mice that reached a particular convulsive stage in case that the number is fewer than the total mice number per group. ***D***, Percent induction of fully generalized kindling in ICER-OE and ICER-KO mice. Only some ICER-OE mice reached a fully kindled state, in contrast to the majority of non-mutant

controls and ICER-KO mice. Compared to non-mutant littermates: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

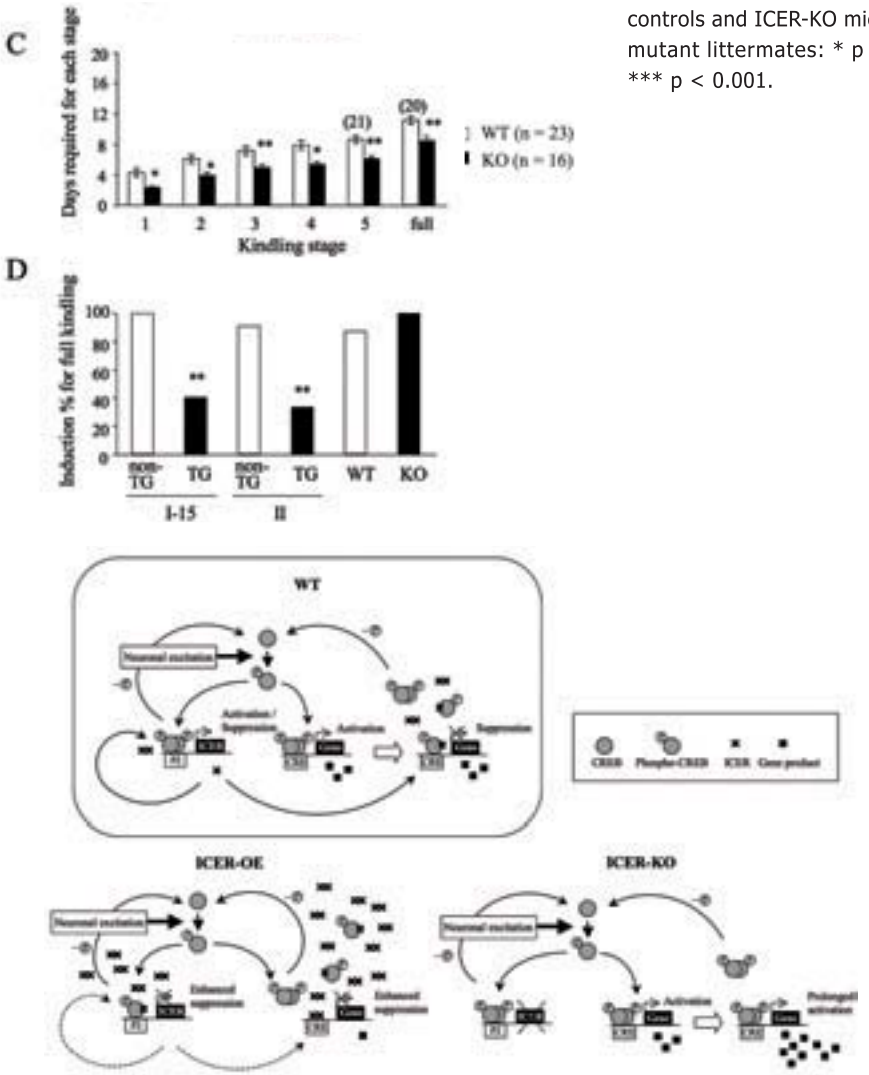

Figure 11. Simplified schematic diagram of ICER involvement in the transcription of CRE-containing genes. In WT mice, phosphorylated CREB binds to the P2 promoter of the CREM/ICER gene and induces ICER expression. ICER forms an ICER homodimer and/or an ICER-CREB heterodimer thus suppress the gene expression. In ICER-OE mice, suppression of the gene transcription is enhanced by an excess amount of ICER. In ICER-KO mice, lack of ICER causes prolonged activation of the gene transcription that results from the loss of an ICER-mediated suppression mechanism. Accumulated gene products resulted from the transcription-translation of the gene is indicated as a closed square. CRE, cAMP responsive element; CREB, cAMP responsive element binding protein; ICER, inducible cAMP early repressor; P, phosphate group; P2, intronic promoter of the CREM/ICER gene.

Reward learning

Most of our behaviors are goal-oriented ones. However, analyses of general behaviors using test battery will not provide the detail of goal-oriented behaviors. To examine the behaviors of genetically-modified mice in detail, we have established the operant conditioning system in mice. Further, we have established the operant conditioning test battery to explore the higher cognitive functions of mice.

Recently it was shown that CREB is involved in the regulation of the depression-like behavior and cocaine reward, suggesting its close involvement in the regulation of the reward learning. CREB modulates excitability of nucleus accumbens neurons and this structure is very important for reward learning and goal-oriented behavior. Possibility of the important role of ICER regulation of the CRE-dependent gene regulation in these types of learning and memory is further, although indirectly, supported by striatopallidal MSN-specific gene Gpr6, that is essential for basal cAMP production, is critical regulator of instrumental conditioning in mice.

Both ICER-OE and ICER-KO mice successfully acquired instrumental responding (lever presses) for cornstarch pellets as reinforcement and learned "non-matching-to-sample" rule at the same level as control animals (Figure 12).

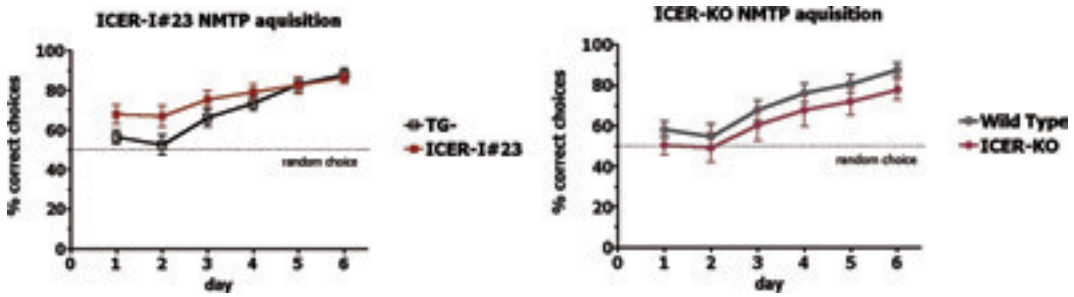

Figure 12. Acquisition of the instrumental Non-Matching-To-Position task in ICER-OE mice (ICER-I #23) and ICER-KO mice. There are tendencies that ICER OE mice learn this task better and ICER-KO mice learn little less. But the differences were not statistically significant.

As a next step, we developed battery of appetitive conditioning tasks to help conduct initial assessment of the different aspects of the reward learning in mutant mice, based on the widely recognized tests developed for rats with modifications.

First we assessed Pavlovian aspects of the appetitive conditioning. Both ICER-OE and ICER-KO mice showed similar to control animals' speed of the Pavlovian conditioning and demonstrated conditioned approach; however, ICER-OE mice failed to show significant conditioned reinforcement and did not show reward devaluation, at the same time, mice lacking ICER (KO) did not differ from control animals in these tests.

On the next stage, mice were shifted to the instrumental conditioning; as in the previous instrumental experiment, both OE and KO did not differ from the respective controls when simple fixed ratio 1 (FR1) or FR1 low variable interval schedules VI (10 s, 30s, 60 s) were implemented. However, ICER-OE mice showed significantly more responses on VI (120s) than non-transgenic littermates, while ICER-KO mice continue to respond on the same level as wild-type controls. Manipulation of ICER level in any direction did not affect Pavlovian-to-instrumental transfer test, although these findings are constrained by the overall weak performance of mice from all groups in this test.

Taken together, our results show that overexpression of ICER impairs some aspects of the reward learning and instrumental conditioning, although further experiments are needed to pinpoint exact stages was affected by gene manipulation. Additional experiments will be performed to reconfirm changes in ICER mice. The preliminary working hypothesis is that the effects of ICER overexpression on appetitive conditioning depend mainly on the changes in "motivation". To examine the hypothesis, instrumental performance will be tested in further operant experiments. In order to gain an insight into the structures and molecular processes involved, western blot/immunohistochemistry analysis focusing on the candidate molecules and structures will be performed after the tasks. The work may provide the opportunity to understand not only the molecular mechanisms for operant conditioning but also those for higher cognitive functions such as motivation. The tasks require the lengthy and labor-intensive period of the preparation before the actual experiments. The preparation includes restriction of food to keep the animal hungry and shaping to push the lever for food. We will further develop the method to run these preparations efficiently and to reduce the labor.

4 Publications

4.1 Journals

Kojima, N., Borlikova, G., Sakamoto, T., Yamada, K., Ikeda, T., Itohara, S., Niki, H., & Endo, S. Inducible cAMP early repressor acts as a negative regulator for kindling epileptogenesis and long-term fear memory. *J Neurosci* 28 (25), 6459-6472 (2008).

Sakamoto, T. & Endo, S. $GABA_A$ receptors in deep cerebellar nuclei play important roles in mouse eyeblink conditioning. *Brain Res* 1230, 125-137 (2008).

Nakazawa, T., Shimura, M., Ryu, M., Nishida, K., Pages, G., Pouyssegur, J., & Endo, S. ERK1 plays a critical protective role against N-methyl-D-aspartate-induced retinal injury. *J Neurosci Res* 86 (1), 136-144 (2008).

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cells. *J Neurosci Res* 87 (6), 1412-1423 (2009).

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4.2 Book(s) and other one-time publications

4.3 Oral presentations

Nakamura, H., Kaneshima, K., Akimoto, M., Takahashi, Y., Endo, S., Saegusa, J., Komura, K. Evaluation of cloth bedding for mouse breeding. The55th Annual Meeting of the Japanese Association for Laboratory Animal Science, Sendai, Japan, May 14-17, 2008

4.4 Posters

Vigot, R., Kojima, N., Noumi, N., Endo, S. Involvement of ICER in the hippocampal long-term potentiation. The 31st Annual Meeting of the Japan Neuroscience Society, Tokyo, Japan, July 9-11, 2008.

Arai, M., Borlikova, G., Kojima, N., Endo, S. Impairment of reward learning in mice overexpressing ICER, an endogenous antagonist of CREB. The 31st Annual Meeting of the Japan Neuroscience Society, Tokyo, Japan, July 9-11, 2008.

Sakamoto, T., Arasaki, T., Endo, S. Neural circuits for mouse eyeblink conditioning: the role of red nucleus and deep cerebellar nuclei under the salient conditioned stimulus. The 31st Annual Meeting of the Japan Neuroscience Society, Tokyo, Japan, July 9-11, 2008.

Rafferty, J. N., Kato, K., Yamada, S., Hirabayashi, Y., Endo, S. Lack of spontaneous activity of cerebellar Purkinje cells in ST3Gal IV KO mice. The 31st Annual Meeting of the Japan Neuroscience Society, Tokyo, Japan, July 9-11, 2008.

Borlikova, G., Arai, M., Kojima, N., Endo, S. Overexpression of ICER an endogenous antagonist of CREB impairs specific aspects of reward learning. The 6th FENS Forum of European Neuroscience, Geneva, Switzerland, July 12-16, 2008.

Sakamoto, T., Arasaki, T., Endo, S. Neural circuits for mouse eyeblink conditioning: the role of red nucleus and deep cerebellar nuclei under the salient conditioned stimulus. The 6th FENS Forum of European Neuroscience, Geneva, Switzerland, July 12-16, 2008.

Rafferty, J. N., Hirabayashi, Y., Endo, S., Kato, K. The loss of sialic acid reduces early-phase long-term depression but does not affect late-phase long-term depression. The 38th Annual Meeting of Society of Neuroscience, Washington D.C., November 15-19, 2008.

Borlikova, G., Arai, M., Kojima, N., Endo, S. Overexpression of ICER facilitates food-reinforced instrumental behaviour on progressive ratio schedule. The 38th Annual Meeting of Society of Neuroscience, Washington D.C., November 15-19, 2008.

Vigot, R., Borlikova, G., Kojima, N., Noumi, N., Endo, S. Overexpression of ICER affects spatial memory but not hippocampal LTP. The 38th Annual Meeting of Society of Neuroscience, Washington D.C., November 15-19, 2008.

Information Processing Biology Unit

Principal Investigator:
Ichiro Maruyama

Research Theme:
Information Processing by Life

Abstract

Our unit interests are (1) to understand how cells/neurons detect external information and transmit it to the inside of the cell and (2) to understand how the nervous system processes the external information as cellular networks to regulate animal behaviors. This year we have made the following findings using cultured animal cells and the nematode *Caenorhabditis elegans* (*C. elegans*) as model systems: (i) We have determined that all the members of the EGF/ErbB receptor family have preformed, yet inactive, homo- and heterodimeric structures between the family members prior to ligand binding. (ii) Cell-surface receptors for neurotrophic factors also function as homo- and heterodimers prior to ligand binding. (iii) In *C. elegans*, mild alkaline pH is sensed by the membrane receptor-type guanylyl cyclase GCY-14 expressed on the surface of the ASEL amphid sensory neuron. The resulting increase of the cGMP concentration in turn opens cGMP-gated calcium channels, encoded by the *tax-2* and *tax-4* genes, for the activation of the ASEL sensory neuron. (iv) We have also found *C. elegans* perceives higher alkalinity than pH 10 as a noxious stimulus. This higher alkaline pH is sensed by OSM-9 (TRPV) channels on ASH sensory neurons as well as by the GCY-35/GCY-36 guanylyl cyclase of the URX chemosensory neurons in the body cavity. These results provide us insights into an understanding of a molecular mechanism underlying information transfer from the outside of cells/neurons to the inside, as well as an understanding of neuronal networks that control animal behaviors in response to external stimuli. These findings may also be invaluable for the development of pharmaceuticals for human diseases such as cancers and mental diseases.

1 Staff

Researchers: Aini Suzana Adenan, Takashi Murayama, Hideki Nagahama, Jianying Shen, Rong-Hua Tao, Zacharie Taoufiq and Qingsheng Yu
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Research administrator: Yuko Toyama

2 Partner Organizations

University of the Ryukyus School of Medicine
Type of partnership: Joint
Name of principal researcher: Ken-ichi Kariya
Research theme: Total internal reflectance fluorescence microscopic analysis of membrane proteins

Niigata University School of Medicine
Type of partnership: Joint
Name of principal researcher: Michihiro Igarashi
Research theme: Studies on roles of axonal growth cone-specific proteins in rat and *C. elegans* nervous systems

3 Activities and Findings

3.1. Project Aims

All forms of life are separated from non-life by cell membranes and all cells/neurons have cell-surface receptor proteins that span the membranes in order to transmit external information, such as environmental changes and cell-cell communications, to the inside of the cell. Such information flow is fundamental for all living systems ranging from bacteria to humans. Dysregulation of the cell surface receptor molecules often causes a variety of impairments including mental and developmental diseases and cancers in humans. (1) We wish to understand at the molecular level how the external information is sensed and transmitted into the inside of cells/neurons by cell-surface receptors and how the information is processed, transferred to other parts of the cells and regulates other cellular activities. (2) We wish also to understand information processing at higher levels through cell-cell communications; namely, how the external information is sensed and transmitted through neuronal cells, processed by the nervous system and how it controls animal behaviors including learning and memory.

3.2. Progress report

3.2.1. Information processing by cells/neurons

3.2.1.1. Background. The bacterial cell-surface receptor Tar recognizes aspartate molecules in the environment and brings bacterial cells toward the higher concentration of the attractant as a nutrient, or the lower concentration of repellents such as nickel and cobalt ions. This transmembrane signaling by Tar occurs within homodimeric receptor molecules on the cell surface (Figure 1A). We have previously shown that the Tar activity is regulated by its ligands, which bind to the extracellular domain of the receptor and lock/freeze the rotational/twist movement of the receptor's transmembrane domains (Maruyama *et al.*, J. Mol. Biol. 253, 530-546 (1995)). The locking/freezing of the rotation/twist at one position by the attractant seems to inhibit the associated histidine kinase Che A, while the locking/freezing at another position by the repellent seems to activate the kinase activity (the rotation/twist model; Figure 1B).

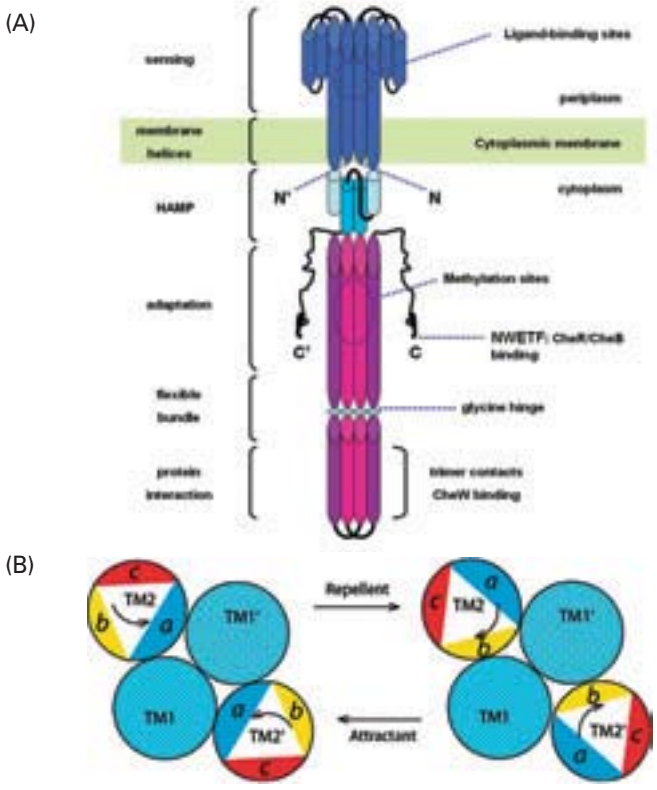


Figure 1. "Rotation/twist" model for the mechanism of the regulation of the Tar receptor activity. (A) Homodimeric structure of Tar. (B) Transverse sections of transmembrane domains of the Tar homodimer. The attractant aspartate locks/freezes the rotation/twist of the transmembrane domain 2 (TM2) by bringing the surface 'a' of TM2 toward TM1, while the repellents bring the surface 'b' of TM2 toward TM1. By contrast, the transmembrane domain 1 (TM1) does not move during the transmembrane signaling.

We have also analyzed a molecular mechanism underlying the activation of the human epidermal growth factor receptor (EGFR) family of cell-surface receptor tyrosine kinases, also known as ErbB or HER. The receptor family consists of four members, EGFR/ErbB1, ErbB2/Neu/HER2, ErbB3/HER3 and ErbB4/HER4, and has a large (~620 amino acid residue long) extracellular ligand-binding region, a single transmembrane α-helix and an intracellular region containing the tyrosine kinase and its regulatory domain. They form a network of homo- and heterodimers. ErbB2 can only be regulated indirectly, and is thought to be the preferred heterodimerization partner for other ErbB receptors. ErbB3, on the other hand, must associate with an ErbB family member that has an active tyrosine kinase in order to respond to its own ligand neuregulin (NRG).

Ligand-induced dimerization has widely been thought to be a property common to the transmembrane signaling mechanism of all known growth factor receptors including the EGF/ErbB receptors (Dimerization Model). According to the model, receptor dimerization is responsible for autophosphorylation of the intrinsic kinase activity, which is mediated by an intermolecular process. Namely, ligand binds to the monomeric form of the receptor and induces its dimeric form for the activation. Prior to ligand binding, however, it still remains controversial whether the receptor has a monomeric or dimeric structure.

We have recently found by chemical cross-linking and sucrose density-gradient centrifugation that in the absence of bound ligand EGFR has an ability to form a dimer and the majority (>80%) of the receptor exists as a preformed dimer on the cell surface. We also analyzed the receptor dimerization by inserting cysteine residues at strategic positions about the α-helix axis of the extracellular juxtamembrane region. The mutant receptors spontaneously formed disulfide bridges and transformed NIH3T3 cells in the absence of ligand, depending upon the positions of the cysteine residues inserted. Kinetic analysis of the disulfide bonding indicated that ligand binding induces flexible rotation or twist of the juxtamembrane region of the receptor in the plane parallel with the lipid bilayer. The binding of an ATP competitor to the intracellular kinase domain also induced similar flexible rotation/twist of the juxtamembrane region. All the disulfide-bonded dimers had flexible ligand-binding domains with the same biphasic affinities for the ligand as the wild type. These results demonstrate that ligand binding to the flexible extracellular domains of the receptor dimer induces rotation/twist of the juxtamembrane regions, hence the transmembrane domains, and rearrange the kinase domains for the receptor activation. Indeed, this rotation/twist model (Figure 2) is consistent with the homodimeric structure of the receptor kinase, transmembrane and unactivated extracellular domains that have recently been determined by others.

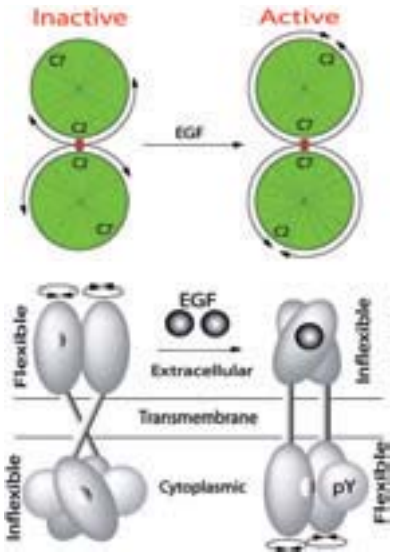


Figure 2. "Rotation/twist" model for the mechanism of the EGFR activation. Helical wheel representation of the transmembrane region of EGFR (Top). Side views of the EGFR dimers (Bottom). EGFR on the cell surface exists as an inactive dimer with flexible ligand-binding domains. The ligand EGF bound to the extracellular domains locks/freezes the flexible extracellular domains, and induces the rotation/twist of the transmembrane domains in parallel with the plane of the plasma membrane, resulting in the dissociation and rearrangement of the intracellular kinase domains for activation.

To support the 'rotation/twist' model, we have recently determined preformed homo- and heterodimeric structures of EGFR and ErbB2 at physiological expression levels (~10^4 molecules per cell), using fluorescence microscopy, fluorescence resonance energy transfer (FRET) and fluorescence cross-correlation spectroscopy (FCCS) (Liu *et al.*, Biophys. J. 93, 684-698 (2007)). When EGFR and ErbB2 fused with a fluorescent protein (FP) were expressed on the cell surface of Chinese hamster ovary cells at physiological expression levels, FRET was detected between the donor and acceptor FPs. Furthermore, cross-correlation between FPs separately fused to EGFR or ErbB2 was also observed by FCCS, indicating that EGFR and ErbB2 molecules diffuse together as homo- or heterodimers in the cell membrane. These results demonstrate that the unactivated cell-surface receptors can spontaneously form homo- and heterodimers, irrespective of their expression levels ranging from ~2 x 10^4 to ~5 x 10^6 molecules per cell.

Thus, different cell-surface receptors, bacterial Tar and human EGF/ErbB receptors, seem to be similarly regulated by their ligands in order to transmit the external information to the inside of the cell. Namely, their ligand binding regulates the rotation/twist of the receptor's transmembrane domain in parallel to the plane of the plasma membrane. Therefore, we are continuing to test the "rotation/twist" model for the activation of other cell-surface receptors including Tar, other ErbB receptors, neurotrophic factor receptors and toll-like receptors as described below.

3.2.1.2. Crystal structure of Tar with a repellent. The three-dimensional structures of the extracellular ligand-binding domain of the bacterial aspartate receptor Tar with or without bound ligands have been determined by others, and only small (~1-2 Å) vertical shift of TM2 from each other has been detected. Based on the 'rotation/twist' model for the molecular mechanism of the Tar regulation (Figure 1B), we have hypothesized that another cofactor for Tar, nickel as a repellent in *E. coli* chemotaxis, may stabilize TM2 in a distinct orientation against TM1. To test this hypothesis, this year we started to produce a large amount of the Tar protein in *E. coli* toward the determination of crystal structure of Tar complexed with the repellent nickel.

3.2.1.3. Preformed homo- and heterodimers between the EGF/ErbB receptor family. We have been analyzing preformed homo- and heterodimeric structures between all the members, EGFR, ErbB2, ErbB3, and ErbB4, of the receptor family by employing bimolecular fluorescence complementation (BiFC) assay and have found that all the members display preformed, yet inactive, homo- and heterodimeric structures in the absence of bound ligand (Tao & Maruyama, J. Cell Sci. 121, 3207-3217 (2008)). The ligand-independent dimerization of the EGF/ErbB receptors occurs in the endoplasmic reticulum (ER) before newly synthesized receptor molecules reach the cell surface. Furthermore, we have also found that ErbB3 was localized in the nucleus when expressed alone or together with ErbB4. When coexpressed with EGFR or ErbB2, however, ErbB3 was located to the plasma membrane (Figure 3). These results indicate that all the EGF/ErbB receptors have homo- and heterodimeric structures before ligand binding, and are consistent with the 'rotation/twist' model (Figures 2 & 4). The ErbB receptors exist as dimers on the cell surface, mainly through interaction between the intracellular kinase domains and C-terminal tails. The receptor dimers have flexible extracellular domains and presumably can take two major conformations with low and high affinities for ligand. Ligand binding to the high affinity receptor stabilizes the extracellular domains, induces approximately 140-degree rotation or twist of the transmembrane domains about its helix axis in parallel to the cell membrane, dissociate the symmetric back-to-back kinase domains and then rearrange the kinase domains to take head-to-tail asymmetric conformation for the receptor activation.

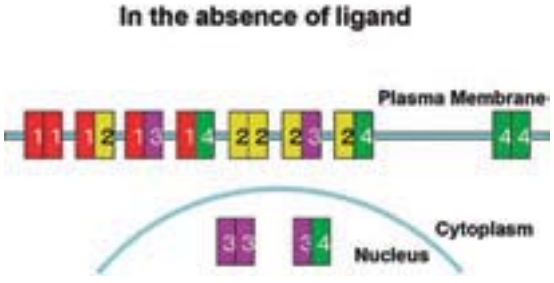


Figure 3. Preformed homo- and heterodimeric structures of the ErbB receptor family and their subcellular localization

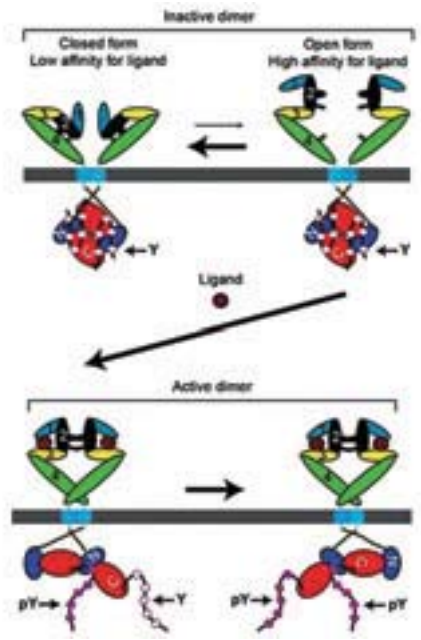
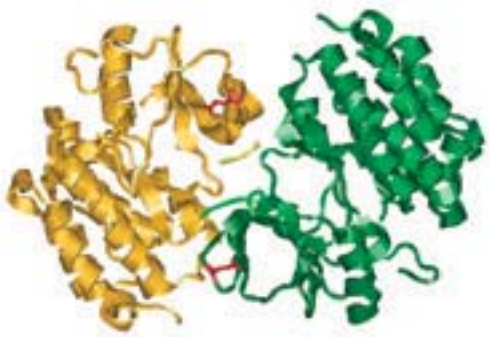


Figure 4. 'Rotation/twist' model for the mechanism of the ErbB receptor activation.

3.2.1.4. EGFR domains required for the homo- and heterodimerization. Three-dimensional structures of the extracellular domains of EGFR and ErbB2 as well as of the intracellular kinase domain of EGFR recently determined by others suggest that the extracellular juxtamembrane region and intracellular kinase domain may play vital roles in the formation of the dimers. Through BiFC analysis of deletion mutants, indeed, we have found that the intracellular domain of EGFR plays a crucial role for the spontaneous dimerization in the absence of ligand (Tao & Maruyama, J. Cell Sci. 121, 3207-3217 (2008)). Based on the three-dimensional structures, we have started systematic analysis of the EGFR domain(s) involved in the spontaneous homo- and heterodimerization. By constructing point and deletion mutant molecules, we are trying to identify domains and amino-acid residues required for the dimerization. When the amino acid residue Glu712 shown in Figure 5 was mutated to lysine, for instance, the kinase was fully activated at the similar level activated by EGF.



(PDB ID 2GS7)

Figure 5. Symmetric structure of the EGFR kinase dimer. The amino acid residues Glu712 are shown in red and C-terminal tail regions involved in the symmetric dimer formation are also indicated in lighter shade compared to their green and yellow kinase domains.

3.2.1.5. Preformed, yet inactive, dimeric structures of receptors for neurotrophic factors. The neurotrophin receptors are thought to be activated through dimerization induced by ligands. Prior to ligand binding, however, it is still unknown whether the receptors exist as monomers, dimers or oligomers on the neuronal cell surface. In the present study, we have analyzed the structures of TrkA, TrkB and p75 receptors for nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-4, and all neurotrophins, respectively, by bimolecular fluorescence complementation (BiFC) assay and luciferase fragment complementation assay. These analyses demonstrated that before ligand binding, the receptors exist as homo- and heterodimers in living cells (Figure 6). Furthermore, the intracellular domains of receptors are necessary for the interaction between two

monomers. Using Brefeldin A to disassemble the Golgi apparatus and block anterograde transport of the receptors from endoplasmic reticulum to Golgi, it was found the preformed dimers were formed in ER. This work provides new insights into understanding of transmembrane signaling by receptors for neurotrophic factors.

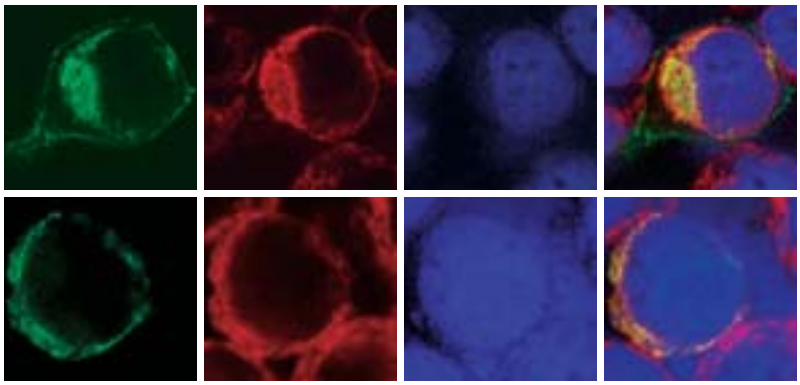


Figure 6. Preformed heterodimeric structure of TrkA and TrkB in endoplasmic reticulum (ER) and on the cell surface. HEK293 cells were co-transfected with pBiFC-TrkA-VC and pBiFC-TrkB-VN plasmids, and cultured in the absence (top row) or presence (bottom row) of Brefeldin A. Venus fluorescence (green) was observed due to TrkA-TrkB heterodimer formation, and ER was detected by immunostaining with anti-calnexin antibody, followed by Alexa Fluor 633-conjugated second antibody (red). Nuclei were also stained with Hoechst 33342 (cyan).

3.2.1.6. Analysis of tertiary structure of toll-like receptors. Toll-like receptors or TLR are an ancient family of receptors that recognize molecular patterns derived from microbes. They have been studied for 20 years having the indispensable role of sensing infections and activating the immune system. However, TLR were shown to be also expressed in the brain, an "immuno-privileged" site. Recently, data have emerged that these receptors may be responsible for brain inflammation processes and thus may be at the "node of the problem" of all neurodegenerative diseases. We are currently studying molecular mechanistics of TLR2 and TLR4 activation.

3.2.2. Information processing by the nervous system

In the past year, we have focused on the analysis of the nematode *C. elegans* chemotaxis toward mild alkaline pH (3.2.2.1.) and away from strong alkaline pH (3.2.2.2), and genome-wide search of lipid-binding proteins in *C. elegans* (3.2.2.3).

3.2.2.1. *C. elegans* chemotaxis toward mild alkaline pH. *C. elegans* responds to water-soluble chemicals such as ions and amino acids. *C. elegans* senses acidic pH as an aversive stimulus and mild alkaline pH as an attractive stimulus. Some of the amphid chemosensory neurons have been shown by neuronal ablation to be responsible for acidic pH avoidance. In general, however, neural mechanisms underlying the alkaline pH response have not been elucidated in any organisms.

To investigate cellular and molecular modes underlying the *C. elegans* chemotactic behavior toward mild alkaline pH, we devised an agar plate assay with a linear pH gradient. Along the gradient from pH 6.8 to pH 8.5, the wild-type worms were attracted toward higher pH regions, whereas *che-1* mutants defective in chemosensory ASE neurons were not (Figure 7A). Since ion sensitivity of a pair of ASE neurons, ASEL (left ASE) and ASER (right ASE), are quite different, we analyzed functions of ASEL and ASER by cell-specific rescue with promoters that specifically drive gene expression in ASEL or ASER. Cell-specific rescue of mutants defective in sensory cilium structure, or cGMP-gated ion channels showed that ASEL function is sufficient for chemotaxis toward mild alkaline pH (Figure 7B). We observed neural activation of ASE in the wild-type animals by using Mermaid, a voltage sensor fluorescent protein, specifically expressed in ASEL. When environmental pH was shifted from pH 7.5 to pH 9.3, a voltage-transient was observed in ASEL, whereas pH shift from pH 9.3 to pH 7.5 had no effect (Figure 7C). This result indicates that ASEL is activated by environmental alkalinization, which is consistent with the results of behavioral assays. On the other hand, in ASER, weak reduction of membrane voltage was observed upon environmental alkalinization. These results indicate that

environmental mild alkaline pH is mainly sensed by ASEL, and cGMP-gated channels in ASEL are required for the neuronal activation.

To search molecular sensors for mild alkaline pH, we have performed a series of RNAi knock-down of genes encoding various channels, cell-surface receptors and guanylyl cyclases in conjunction with analysis of chemotaxis mutants. Among the genes analyzed, *tax-2*, *tax-4* and *gcy-14* have been found to be involved in the *C. elegans* chemoattraction toward mild alkaline pH (Figure 7A, D). It is known that these three genes are expressed in ASEL. GCY-14 is homologous to mammalian receptor-type guanylyl cyclase, GC-G, which is abundant in the testis and sperm. These results indicate that GCY-14, a membrane receptor-type guanylyl cyclase, functions as a mild alkaline pH sensor on the surface of amphid chemosensory ASEL neuron. A rise of environmental pH activates GCY-14 to produce cGMP in ASEL. An increase of cGMP concentration in turn opens the TAX-2/TAX-4 calcium channel to activate the ASEL sensory neuron.

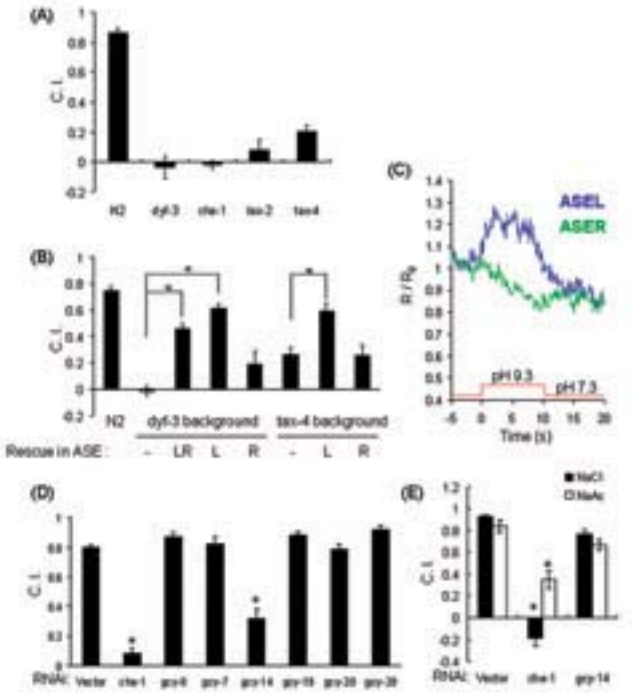


Figure 7. (A) Chemotaxis to mild alkaline pH of the wild-type N2 strain and mutants defective in chemosensation. C. I. denotes chemotaxis index. (B) Chemotaxis to mild alkaline pH of *dyf-3* or *tax-4* mutant animals rescued by cell-specific gene expression. L and R indicate that the mutants were rescued by specifically expressing the respective gene in ASEL or ASER. (C) Voltage-transients in ASEL or ASER depended on environmental alkalinization. R is the fluorescence intensity ratio of orange over green emitted from the Mermaid fluorescent protein. R_0 is R at -0.5 s. Red line indicates up- or down-shift of pH. Mild alkaline solution, pH 9.3, was applied at 0 s. Blue and green represent the mean value of voltage-transients. (D) Chemotaxis to mild alkaline pH of RNAi-treated animals. Note that knockdown of the *che-1* or *gcy-14* gene lowered their chemotactic activity. (E) Chemotaxis along the NaCl or sodium acetate (NaAc) gradient of RNAi-treated animals. Bars, SEM. * : $p < 0.001$ in ANOVA.

3.2.2.2. Nociception in *C. elegans*. We have previously found *C. elegans* avoids ammonia gas. However, we do not know whether the worm senses the gas molecules or extremely higher pH as a noxious stimulus. To elucidate a molecular mechanism underlying the nociception in *C. elegans*, we designed the similar plate assay to the mild alkaline chemotaxis assay described above. An ASE-defective *che-1* mutant avoided higher pH ranges than pH 10.0. These suggest that while ASE neurons may be required for chemotaxis toward mild alkaline pH, some other neurons than ASE may be responsible for avoidance of the higher alkaline pH. After analysis of known chemotaxis mutants, we have found that *C. elegans* mutants defective in *osm-9*, *gcy-35* and *gcy-36* cannot avoid the higher alkaline pH in the plate assay. Cell-specific rescue experiments suggested that these genes are working in ASH amphid chemosensory neurons and URX sensory neurons in the body cavity. We are currently trying to identify sensor molecules for the higher pH ranges and neuronal networks responsible for the nociception and avoidance behavior using the paradigm we developed.

3.2.2.3. Genome-wide analysis of lipid-interacting proteins by using lambda phage surface display. In order to systematically search for proteins that have domains or motifs that recognize membrane lipids in *C. elegans*, we have chosen λ phage surface display (Figure 8), which can express foreign proteins on the phage particle surface (Maruyama *et al.*, Proc. Natl. Acad. Sci USA 91, 8273-8277 (1994); Mikawa *et al.*, J. Mol. Biol. 262, 21-30 (1996)). Using lipid as a probe, the phage cDNA libraries can specifically be searched for phage clones encoding lipid-interacting proteins through several rounds of affinity selections. Toward the goal, we have made a series of λ foo constructs which express domains that have been reported to interact with lipids to define experimental conditions for the affinity screening.

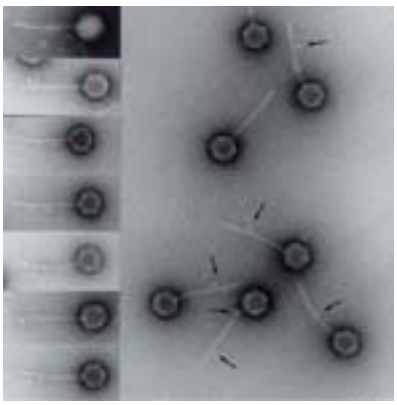


Figure 8. Lambda phage particles expressing beta-galactosidase tetramers on their tails.

4 Publications

4.1 Journals

Maruyama, I. 'Rotation/twist' model for the activation of the EGF/ErbB receptor family. *Mol. Biol. Cell* 19 (Suppl), 473 (CD-ROM) (2008).

Tao, R.H. & Maruyama, I.N. All EGF(ErbB) receptors have preformed homo- and heterodimeric structures in living cells. *J Cell Sci* 121, 3207-3217 (2008).

Tao, R.H. & Maruyama, I.N. Ligand-induced activation of preformed inactive EGF/ErbB receptor homo- and heterodimers: a model for EGF/ErbB receptors. *FASEB Journal* 22:1054.1 (2008).

Maruyama, I.N. 'Rotation/twist' model for the EGF/ErbB receptor family activation. *FASEB Journal* 23, 884.2 (2009).

4.2 Book(s) and other one-time publications

None

4.3 Oral presentations

Maruyama, I. Transmembrane signaling by cell-surface receptors., Institute of Molecular and Cellular Biosciences, University of Tokyo, Tokyo, Japan, July 8, 2008

Murayama, T., Maruyama, I. Neurons responsible for *C. elegans* chemosensation to alkaline pH. OIST Junior Researcher Retreat '08, Seaside House, Onna, Okinawa, Japan, October 30-31, 2008

Maruyama, I. Observing a single molecule on the cell surface., OIST open house 2008, Okinawa Industrial Technology Center, Uruma, Okinawa, Japan, November 9, 2008

Maruyama, I. Transmembrane signaling by cell-surface receptors., Undergraduate Program, Ryukyu University School of Medicine, Nishihara, Okinawa, Japan, January 8, 2009

Maruyama, I. Around the world in 23 years as a scientist., Ishikawa High School, 861 Iha, Ishikawa, Uruma, Okinawa, Japan, January 29, 2009

Maruyama, I. Rotation/twist' model for the mechanism of the ErbB receptor family activation Experimental Biology 2009, Ernest N. Morial Convention Center, New Orleans, Louisiana, USA April 18-22, 2009

4.4 Posters

Tao, R.-H., Maruyama, I. N. Ligand-induced activation of preformed inactive EGF/ErbB receptor homo- and heterodimers: a model for EGF/ErbB receptors, ASBMB 2008 Annual Meeting in conjunction with Experimental Biology 2008, San Diego Convention Center, San Diego, California, USA, April 5-9, 2008

Nagahama, H., Maruyama, I. Towards genome-wide analysis of lipid-interacting proteins by using lambda phage surface display, The East Asia Worm Meeting 2008, Shanghai Jiatong University School of Medicine, Shanghai, China, April 18-21, 2008

Adenan, A. S., Maruyama, I. Role of amino acid residue 712 in EGFR activation., OIST Junior Researcher Retreat '08, Seaside House, Onna, Okinawa, Japan, October 30-31, 2008

Miyagi, H. Maruyama, I. FCS measurement of dissociation constants between EGF and EGFR on living cells, OIST Junior Researcher Retreat '08, Seaside House, Onna, Okinawa, Japan, October 30-31, 2008

Nagahama, H., Maruyama, I. Towards genome-wide analysis of lipid-interacting proteins by using lambda phage surface display, OIST Junior Researcher Retreat '08, Seaside House, Onna, Okinawa, Japan, October 30-31, 2008

Adenan, A. S., Miyagi, H., Maruyama, I. Unraveling the role of preformed epidermal growth factor receptor dimers by mutagenesis analysis, OIST International Workshop "Gradients and Signalling: from chemotaxis to development," Seaside House, Onnna, Okinawa, Japan, November 17-21, 2008

Murayama, T., Maruyama, I. Molecular mechanism underlying *C. elegans* chemotaxis toward alkaline pH, OIST International Workshop "Gradients and Signalling: from chemotaxis to development," Seaside House, Onnna, Okinawa, Japan, November 17-21, 2008

Nagahama, H., Maruyama, I. Towards genome-wide analysis of lipid-interacting proteins by using λ phage surface display, OIST International Workshop "Gradients and Signalling: from chemotaxis to development," Seaside House, Onnna, Okinawa, Japan, November 17-21, 2008

Shen, J., Maruyama, I. Preformed, yet inactive, dimeric structures of receptors for neurotrophic factors. OIST International Workshop "Gradients and Signalling: from chemotaxis to development," Seaside House, Onnna, Okinawa, Japan, November 17-21, 2008

Shen, J., Maruyama, I. Preformed, yet inactive, dimeric structures of receptors for neurotrophic factors, BMB2008 (81st Annual Meeting of the Japanese Biochemical Society and 31st Annual Meeting of the Molecular Biology Society of Japan), Kobe Port Island, Kobe, Japan, December 9-12, 2008

Fujiwara, M., Sanehisa, S., Murayama, T., Maruyama, I. *C. elegans* mutants defective in avoidance behaviors from strong-alkaline pH, BMB2008 (81st Annual Meeting of the Japanese Biochemical Society and 31st Annual Meeting of the Molecular Biology Society of Japan), Kobe Port Island, Kobe, Japan, December 9-12, 2008

Murayama, T., Maruyama, I. Molecular mechanism underlying *C. elegans* chemotaxis toward alkaline pH, BMB2008 (81st Annual Meeting of the Japanese Biochemical Society and 31st Annual Meeting of the Molecular Biology Society of Japan), Kobe Port Island, Kobe, Japan, December 9-12, 2008

Miyagi, H., Adenan, A. S., Maruyama, I. Dissociation constants between EGF and EGFR on living cells using fluorescence correlation spectroscopy (FCS), BMB2008 (81st Annual Meeting of the Japanese Biochemical Society and 31st Annual Meeting of the Molecular Biology Society of Japan), Kobe Port Island, Kobe, Japan, December 9-12, 2008

Maruyama, I., Rotation/twist' model for the activation of the EGF/ErbB receptor family, 48th Annual Meeting of the American Society for Cell Biology, Moscone Center, San Francisco, California, USA December 13-17, 2008

Maruyama, I. Rotation/twist' model for the mechanism of the ErbB receptor family activation Experimental Biology 2009, Ernest N. Morial Convention Center, New Orleans, Louisiana, USA April 18-22, 2009

5 Intellectual Property Rights and Other Specific Achievements

Nothing to report.

6 Meetings and Events

6.1 Seminars hosted

"Migration of epithelia: The mechanobiology of cells and tissues"
Date: April 7, 2009
Venue: OIST IRP Conference Room
Speaker: Paul Matsudaira, Department of Biological Sciences,
National University of Singapore, Singapore

"The Broadest panel of kinase solutions for characterization, profiling & screening"
Date: March 19, 2009
Venue: OITC 2F Salon Room
Speaker: Taffeta Chen, DiscoveRx Corp. Tokyo, Japan

"Regulation of gonad formation by chondroitin sulfate proteoglycan, and characterization of molecule involved in neural gene expression in *C. elegans*"
Date: March 2, 2009
Venue: OITC 2F Salon Room
Speaker: Toshihiro Sassa, Center for Developmental Biology, Kobe, Japan

"The novel neuronal growth-associated proteins revealed by the proteomic analysis of the growth cone"
Date: February 5, 2009
Venue: OITC 2F Salon Room
Speaker: Michihiro Igarashi, Division of Molecular and Cellular Biology,
Graduate School of Medical and Dental Sciences, Niigata University, Niigata, Japan

"Interaction and localization of Necl-5 and PDGF receptor beta at the leading edges of moving NIH3T3 cells: Implications for directional cell movement"
Date: November 27, 2008
Venue: OITC 3F Conference Room
Speaker: Hiroyuki Amano, Kobe University Graduate School of Medicine, Kobe, Japan

"Fabrication and application of lab-on-a-chip system"
Date: July 16, 2008
Venue: OITC 2F Salon Room
Speaker: Richard Jongzen Huang, Hua-Fan University, Taipei, Taiwan

"Elephant shark genome provides insights into the evolutionary history of vertebrate genomes"
Date: June 13, 2008
Venue: OITC 2F Lecture Hall
Speaker: Byrappa Venkatesh, Institute of Molecular and Cell Biology, Singapore

6.2 Workshop co-organized

"Gradients and signaling: from chemotaxis to development"

Date: November 17-21, 2008

Venue: OIST Seaside House, Onna, Okinawa, Japan

Co-organizers: Ichi Maruyama, Ichiro Masai, Mary Ann Price, and Fadel Samatey

Co-sponsors: Okinawa Institute of Science and Technology, Carl Zeiss Microimaging, Okinawa Medix, and Tomy Okinawa Novo Science

Speakers:
1) Phil Beachy, Stanford University, USA
2) Sudipto Roy, IMCB, Singapore
3) Matthew Scott, Stanford University, USA
4) James Briscoe, NIMR, London, UK
5) Konrad Basler, University of Zurich, Switzerland
6) Caroline Hill, CRUK, London, UK
7) Hiroshi Hamada, Osaka University, Japan
8) Ken Yamaguchi, University of Southern California, USA
9) Osamu Shimmi, University of Helsinki, Finland
10) Zhaohui Wang, Chinese Academy of Sciences, Beijing, China
11) Xi He, Harvard Medical School, Boston, USA
12) Shinji Takada, National Institutes of Natural Sciences, Okazaki, Japan
13) Tetsuya Tabata, University of Tokyo, Japan
14) Wenqing Xu, University of Washington, Seattle, USA
15) Jean-Paul Vincent, NIMR, London, UK
16) John S. Parkinson, University of Utah, Salt Lake City, USA
17) Ikuro Kawagishi, Hosei University, Tokyo, Japan
18) Ariane Briegel, California Institute of Technology, Pasadena, USA
19) Victor Sourjik, University of Heidelberg, Germany
20) Brian Crane, Cornell University, Ithaca, USA
21) Yasunobu Igarashi, Tohoku University, Japan
22) Jasmine Plummer, University of Toronto, Canada
23) Mu-ming Poo, University of California, Berkeley, USA
24) Joseph Culotti, University of Toronto, Canada
25) Eric Theveneau, University of College London, UK
26) Matthew Freeman, MRC-LMB, Cambridge, UK
27) Rizaldy Scott, Samuel Lunenfeld Research Institute, Toronto, Canada
28) Maria Leptin, University of Cologne, Germany
29) Shin-ichiro Takahashi, University of Tokyo, Japan

Developmental Neurobiology Unit

Principal Investigator:
Ichiro Masai

Research Theme:
Mechanisms underlying neuronal differentiation in the zebrafish retina

Abstract

In the vertebrate retina, six major classes of retinal neurons differentiate and form a relatively simple neural circuit, which is responsible for phototransduction and visual processing. In the developing vertebrate retina, cell fate decision is independent of cell lineage, and multipotent neural progenitor cells generate all retinal cell types. However, the molecular mechanism underlying the generation of diverse retinal cell types remains unclarified. To elucidate this mechanism, we study retinal development in the zebrafish (*Danio rerio*). First, we focused on the initial step of neuronal differentiation, that is, neurogenesis. In the developing zebrafish retina, neurogenesis is initiated at a few cells adjacent to the optic stalk and progresses into the entire neural retina. We found that two signaling molecules, Hedgehog (Hh) and Fibroblast growth factor (FGF), regulate the initial induction and progression of retinal neurogenesis in zebrafish, respectively. Furthermore, we found that downstream of these signaling pathways, Histone deacetylase 1 (HDAC1) regulates retinal neurogenesis by suppressing both Wnt and Notch signaling pathways, which promotes cell proliferation and neurogenic inhibition, respectively. One of the aims of our research is to elucidate the molecular mechanism that regulates the pattern of retinal neurogenesis in zebrafish. Furthermore, we performed a large-scale mutagenesis using zebrafish and identified more than 300 zebrafish mutants that show various defects in retinal development, for example, apoptosis of differentiating retinal neurons, disorganization of retinal layers, photoreceptor degeneration, and disorganized lens fiber formation. To understand the later steps of retinal cell differentiation as well as the pathological processes of retinal and lens diseases, we currently characterize these mutant phenotypes and clone their mutant genes.

1 Staff

Developmental Neurobiology Unit

Researchers: Dr. Yuko Nishiwaki
Dr. Toshiaki Mochizuki
Dr. Fumiyasu Imai
Dr. Yukihiro Yoshimura

Technical Staff: Ms. Nozomi Hanahara
Dr. Masaya Morita
Ms. Asuka Yoshizawa

Research Administrator / Secretary: Ms. Ayako Gima

2 Partner Organizations

3 Activities and Findings

3.1 Mechanism regulting the spatial and temporal pattern of retinal neurogenesis in zebrafish

In the developing zebrafish retina, neurogenesis is initiated at a few cells adjacent to the optic stalk and progresses to the entire neural retina. Such a pattern of retinal neurogenesis provides a good model for studying the mechanism regulating the spatial and temporal pattern of neurogenesis in the nervous system. Previous studies including ours suggest that at least five signaling molecules regulate the pattern of retinal neurogenesis in zebrafish. Fibroblast growth factors (Fgfs) are expressed in the optic stalk and required for the initial induction of retinal neurogenesis in zebrafish. The Hedgehog signaling pathway is important for the progression of retinal neurogenesis in zebrafish. The activation of Wnt and Notch signaling pathways promotes cell proliferation and inhibits neurogenesis in the zebrafish retina, respectively. Previously, we identified a zebrafish mutant, namely, *ascending and descending (add)*, in which retinal progenitor cells fail to exit from the cell cycle and continue to proliferate (Fig. 1). We showed that the *add* mutant gene encodes histone deacetylase 1 (HDAC1), which promotes retinal neurogenesis by suppressing both Wnt and Notch signaling pathways. HDAC1 is recruited to several transcription repressor and corepressor complexes, suggesting that HDAC1 interacts with signaling pathways other than the Wnt and Notch pathways. To elucidate the HDAC1-dependent regulation of retinal neurogenesis, we are currently identifying factors interacting with HDAC1 and its downstream targets. As a genetic approach, we searched new mutations that modify the hyperproliferation of retinal cells in the *add* mutant. To date, we have screened 72 mutagenized genomes and identified a mutation, namely *oki150*, that enhances the *add*-mediated hyperproliferation in heterozygous. Because it is possible that the protein encoded by the *oki150* mutant gene interacts with HDAC1 to promote retinal neurogeneis in zebrafish (Fig. 2), we mapped the *oki150* mutational locus on zebrafish chromosome 21 and are currently cloning the *oki150* mutant gene.

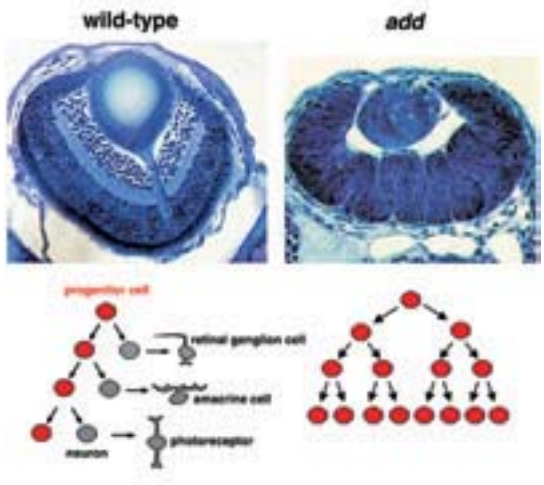


Fig.1 *add* mutant retina at 2dpf.
In the *add* mutant, retinal progenitor cells (red) fail to exit from the cell cycle and continue to proliferate.

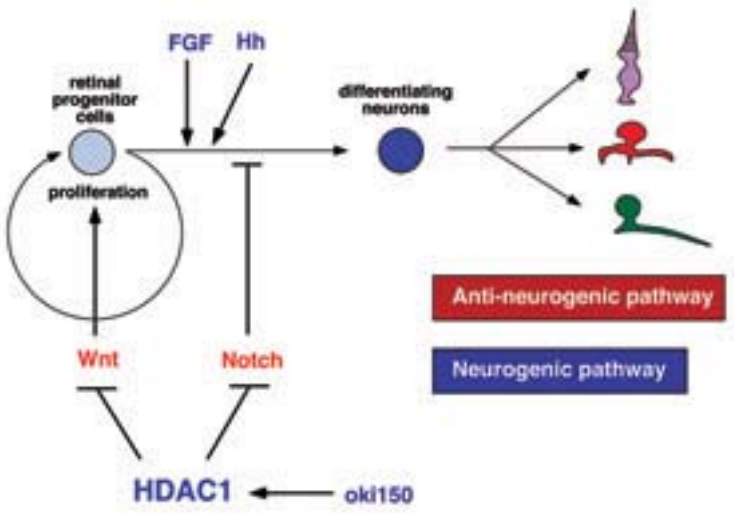


Fig.2 Molecular network regulating retinal neuroegnesis in zebrafish.
FGF and Hh regulates the induction and progression of retinal neurogenesis in zebrafish. Wnt and Notch signaling pathways promote cell proliferation and neurogenic inhibition, respectively. HDAC1 promotes retinal neurogenesis by suppressing both Wnt and Notch signaling pathways. A novel mutation namely *oki150* may interact with HDAC1 to promote neurogenesis.

3.2 Mechanism regulating apoptosis during retinal development in zebrafish

Apoptosis is often observed in a developing tissue including the retina, and is believed to remove abnormal cells such as cancer-predisposing cells. Although such an apoptosis-mediated elimination of abnormally differentiated neurons may be important for the establishment of precise neural circuits in the nervous system, it is essentially unknown how the apoptotic pathway is regulated during neurogenesis. Previously, we performed a large-scale mutagenesis using zebrafish and identified four zebrafish mutants, which show severe apoptosis of differentiating retinal neurons. In these mutants, differentiating retinal neurons are selectively eliminated prior to their maturation, but retinal stem cells seem intact, suggesting that the switch between differentiation and apoptosis seems deregulated. To understand the molecular network of retinal apoptosis, we characterized one of these apoptotic mutants, *pinball eye* (*piy*). We found that the *piy* mutant gene encodes the small subunit of DNA primase (Prim1). Prim1 is essential for DNA replication and also required for the activation of the DNA damage response, which may activate a PI3-kinase family protein, Ataxia telangiectacia mutated (ATM). ATM activates Checkpoint kinase 2 (Chk2), and Chk2 subsequently activates the tumor suppressor p53, which repairs damaged DNA or, if not, induces apoptosis. We found that apoptosis in the *piy* mutant depends on the ATM-Chk2-p53 apoptotic pathway, suggesting that aberrant activation of the DNA damage response and p53 causes severe apoptosis in the *piy* mutant retinas. Interestingly, the *piy* mutant retinal cells can differentiate into fully functional retinal neurons in the absence of p53 activity. Taken together, these findings suggest that the DNA damage response and its downstream target p53 determine whether retinal cells will differentiate normally or undergo apoptosis during retinal neurogenesis.

Fig.3 Zebrafish piy mutant shows severe apoptosis of retinal neurons.
(A) Zebrafish *piy* mutant. Severe apoptosis is observed in retina and tectum (arrowheads).
(B, C) Three dpf wild-type (B) and *piy* mutant (C) retinas. In the *piy* mutant, retinal stem cells normally proliferate (asterisks), but almost retinal neurons undergo apoptosis and are eventually eliminated.

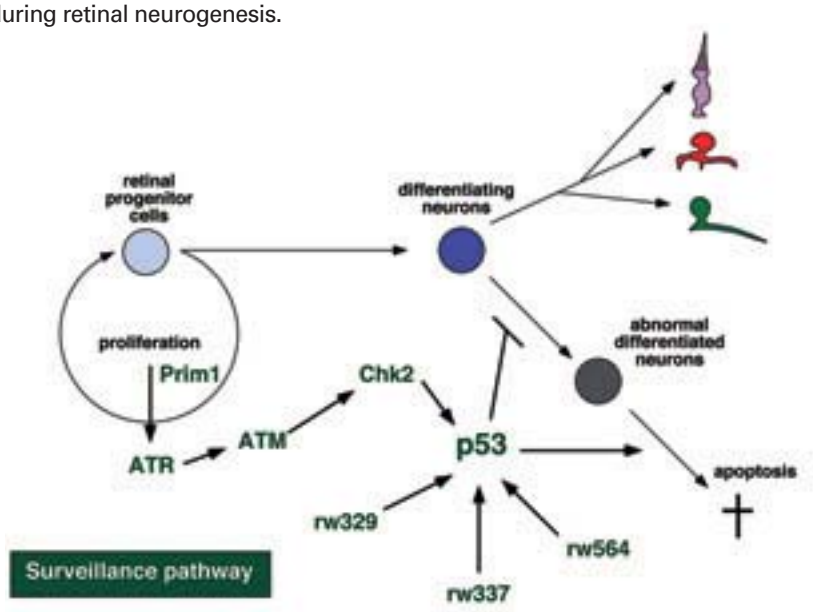


Fig.4 Surveillance pathway in the developing zebrafish retina. Prim1 activates DNA replication checkpoint ATR. ATR activates the DNA damage response, ATM and Chk2. Chk2 activate p53, which repair DNA damages or induce apoptosis to eliminate abnormal differentiated cells

In addition to the *piy* mutant, we characterized retinal phenotypes of three other apoptotic mutants, namely, *rw329*, *rw337*, and *rw564*. We found that retinal apoptosis in all these mutants depends on p53, but not on Chk2. These observations suggest that the DNA damage response is not the only pathway that activates p53 in the zebrafish retina (Fig. 4). To elucidate the p53-dependent molecular mechanism that determines whether retinal cells are eliminated by apoptosis, we mapped these three genetic loci on zebrafish chromosomes and are currently cloning them.

3.3 Roles of apicobasal cell polarity in retinal neurogenesis

Cell and epithelial polarity is severely compromised in various human cancers. It has been reported that the loss of apicobasal cell polarity and the disruption of adherens junctions induce hyperplasia in the mouse developing brain and *Drosophila* imaginal discs. However, the relationship among the loss of cell polarity, proliferation, and differentiation has been discussed for a long time. Three groups of proteins play a central role in the establishment and maintenance of apicobasal cell polarity. The Crumbs-Pals1 (Stardust)-Patj and Par3 (Bazooka)-Par6-aPKC protein complexes localize to the apical membrane domain and promote apical-membrane domain identity. Their function is antagonized by the basolaterally localized Lethal giant larvae (Lgl), Scribble (Scrib), and Discs large (Dlg) proteins, which together promote basolateral membrane identity. In the epithelial tissue, the apical and basolateral membrane domains are separated by a physical barrier called the apical junctional complex, which comprises tight junctions and adherens junctions and functions as a crucial physical link between internal cell polarity and three-dimensional tissue organization.

To elucidate the role of cell polarity and epithelial integrity in retinal neurogenesis, we examined retinal phenotypes in zebrafish *n-cadherin* (*ncad*) and *nagie oko* (*nok*) mutants, in which the apicobasal cell polarity and adherens junctions in the retinal epithelium are severely disrupted. We found that the ratio of the number of proliferating cells to the total number of retinal cells is higher in these mutants during neurogenic stages, suggesting hyperplasia associated with retinal neurogenesis. We observed that the cell-cycle progression is not activated but the rate of neurogenesis is significantly reduced in these mutant retinas. These observations suggest that the inhibition of cell-cycle exit is a primary defect of such a hyperplasia-like phenotype in these mutant retinas. Furthermore, we found that the switching from proliferative cell division to neurogenic cell division is compromised in these mutant retinas. Taken together, these observations suggest that the loss of apicobasal cell polarity and the disruption of epithelial integrity affect the regulation of the mode of cell division during neurogenesis in the zebrafish retinas.

3.4 Mechanism regulating structural and functional integrities of photoreceptors

There are hereditary retinal diseases in humans, for example, retinitis pigmentosa, in which photoreceptor cells degenerate. To date, about 185 genetic loci associated with retinal diseases have been mapped to human chromosomes. The human genome project accelerated the cloning of mutant genes, and more than 100 genes have been identified (see Ret Net *http://www.sph.uth.tmc.edu/Retnet*). These genes function in signaling pathways involved in various biological aspects of photoreceptors: phototransduction, retinoid metabolism, and protein transport within photoreceptors. However, there are still many molecules whose functions are unclarified.

To elucidate the mechanisms underlying the structural and functional integrities of photoreceptors, we previously screened and identified zebrafish mutants with disrupted

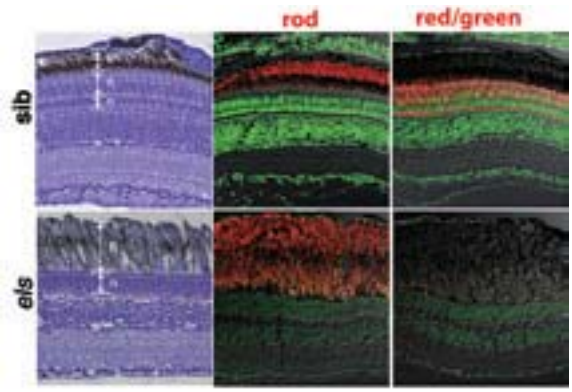


Fig.5 Progressive degeneration of cones in the els mutant.
Wild-type sibling (upper panels) and the *els* mutant (lower panels) retinas at the adult stage. Plastic sections (left panels) and labeling with anti-rhodopsin antibody (red, middle panels) and zpr-1 antibody, which stains double cones (red, right panels). In the *els* mutant, cones are absent (red, right-lower panel), but rods seem to increase in number (red, middle-lower panel).

visual behaviors such as optokinetic response (OKR). The *eclipse* (*els*) mutant is one of such OKR-defective mutants. We found that the *els* mutant gene encodes cGMP-phosphodiesterase 6α'-subunit (PDE6α'). PDE6 is an enzyme that mediates phototransduction in vertebrate photoreceptors, rods and cones. Rod PDE6 is composed of two catalytic subunits, α and β, and two inhibitory γ subunits, whereas cone PDE6 consists of two identical catalytic α' subunits complexed with the two inhibitory γ subunits. The *els* mutant displays no visual response to light, where cones are active, but shows relatively normal OKR to dim light, where only rods function. These observations suggest that only the cone-specific phototransduction pathway is disrupted in the *els* mutant. In human genetic diseases associated with photoreceptor degeneration, mutations in the PDE6 α and β subunit genes have been reported. However, no mutation in PDE6α' genes have been found in humans. To elucidate whether photoreceptor degeneration occurs in the absence of PDE6α' activity, we examined the morphology of the *els* mutant retinas during development. We found that cones are selectively eliminated but rods are retained at the adult stage in the *els* mutant, suggesting that cones undergo a progressive degeneration in the *els* mutant retinas (Fig. 5). Taken together, these findings suggest that PDE6α' activity is important for not only cone-specific phototransduction but also the survival of cones in zebrafish.

3.5 Mechanism regulating the differentiation of lens fibers

Lens is an intraocular tissue, which focuses rays of light on retinal photoreceptor layer. During development, lens precursor region is initially induced in epidermal ectoderm overlying neuroectoderm destined to the retinal primordium, and invaginates into the pocket of the optic cup to form the lens vesicle. After the lens vesicle formation, cells in the posterior epithelium of the lens vesicle differentiate into lens fiber cells, which form a spherical core of lens fibers. On the other hand, cells in the anterior epithelium become mitotic epithelial cells and cover the anterior half of the lens fiber core. At the equatorial zone of the lens vesicle, which corresponds to the interface between the anterior lens epithelium and the posterior lens fiber core, epithelial cells exit from the cell cycle and start to differentiate into lens fiber cells. Newly differentiating lens fiber cells elongate through a bi-directional extension toward anterior and posterior poles of the spherical lens core, and cover the old lens fiber core like an onion (Fig. 6AB). The developmental profile of the vertebrate lens provides a good model in which to study the mechanism underlying the maintenance of mitotic stem cells, the switch from proliferation to differentiation, and cell morphogenesis associated with cell differentiation. In addition, the lens fiber differentiation is associated with the elimination of internal organelles including the nucleus and endoplasmic reticulum. Although the loss of organelles during lens differentiation is important for the acquisition of its transparency, the mechanism underlying organelle elimination during lens fiber differentiation is largely unknown.

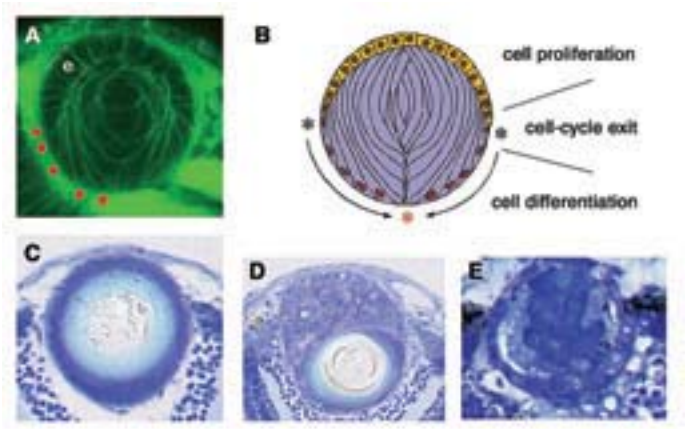


Fig.6 Zebrafish developing lens
(A) 30 hpf zebrafish lens labeled with a fluorescence-conjugated lipid. e; lens epithelial cells. Red circles indicate basal ends of differentiating lens fiber cells. (B) Schematic drawing of lens fiber cells. Yellow and light blue colors indicate lens epithelial cells and differentiating lens fiber cells, respectively. Black asterisks indicate the equatorial zone of the lens sphere, where lens epithelial cells exit from the cell cycle. Red asterisk indicates a suture point where the basal ends of opposing lens fibers meet and intercalate. (C, D) Five dpf wild-type and *rw341* mutant lens. In the *rw341* mutant, anterior lens epithelial cells overgrow like a cancer. (E) Three dpf *vov* mutant lens. Nuclei fail to be eliminated in the *vov* mutant lens.

To elucidate these mechanisms, we focus on zebrafish mutants showing the defects in lens fiber differentiation. In our zebrafish mutant screening, we identified two zebrafish lens-defective mutants, namely, *rw341* and *volvox* (*vov*). In the *rw341* mutant, lens epithelial cells lose their adhesion and overgrow like a cancer, resulting in severe disorganization of anterior lens epithelium (Fig. 6CD). In the *vov* mutant, differentiating lens fiber cells do not differentiate into mature lens fibers, and their

nuclei frequently fail to be eliminated (Fig. 6E). These findings suggest that the proteins encoded by the *rw341* and *vov* mutant genes are required for structural integrity of the lens epithelium and lens fiber differentiation, respectively. We mapped these mutations on zebrafish chromosomes and are currently cloning these mutant genes.

4 Publications

4.1 Journal Articles

Yamaguchi, M., Fujimori-Tonou, N., Yoshimura, Y., Kishi, T., Okamoto, H., & Masai, I. Mutation of DNA primase causes extensive apoptosis of retinal neurons through the activation of DNA damage checkpoint and tumor suppressor p53. *Development* 135 (7), 1247-1257 (2008).

Nishiwaki, Y., Komori, A., Sagara, H., Suzuki, E., Manabe, T., Hosoya, T., Nojima, Y., Wada, H., Tanaka, H., Okamoto, H., & Masai, I. Mutation of cGMP phosphodiesterase 6alpha'-subunit gene causes progressive degeneration of cone photoreceptors in zebrafish. *Mech Dev* 125 (11-12), 932-946 (2008).

4.2 Book(s) and other one-time publications

Masai, I. Mechanism that induces and ensures retinal neurogenesis in zebrafish, *Syujyunsya Co. Ltd./Cell technology* 28, 52-58 2008

4.3 Oral presentations

Ohata, S., Kinoshita, S., Tsuruoka, S., Tanaka, H., Wada, H., Masai, I., Okamoto, H. Role of holm/mosaic eyes, a neuroepithelial polarity gene in the migration of vagus motor neuron precursors., 8th Inthernational conference on zebrafish development and genetics, Madison, USA, June 25-29, 2008

Masai, I. Mechanism underlying regional and neuronal specification in the retina and epiphysis., 79th Annual meeting of the zebrafish society of Japan, Fukuoka, Japan, September 5-7, 2008

Masai, I. Roles of DNA damage checkpoint and p53 in retinal neurogenesis in zebrafish The 2nd CNU International symposium on Biology, Daejeon, Korea, February 17, 2009

Yoshimura, Y., Hanahara, N., Fujimori, N., Yamaguchi, M. Okamoto, H., Masai, I. Analysis of novel zebrafish mutants showing p53-dependent apoptosis of retinal neurons., 31th Annunal meeting of Japanese society of Molecular biology, December 9-12, 2008

4.4 Posters

Nishiwaki, Y., Komori, A., Sagara, H., Suzuki, E., Hosoya, T., Okamoto, H., Masai, I. Mutation of cGMP phosphodiesterase 6 alpha'-subunit gene causes progressive degeneration of cone photoreceptors in zebrafish, 8th International conference on zebrafish development and genetics, Masidon, USA, June 25-29, 2008

Imai, F., Yoshizawa, A., Okamoto, H., Masai, I., Analysis of zebrafish mutants showing defects in lens fiber differentiation, 31th Annual meeting of Japanese society of Molecular Biology December 9-12, 2008

5 Intellectual Property Rights and Other Specific Archivements

6 Meetings and Events

6.1 Workshop: Gradients and signaling: from chemotaxis to development

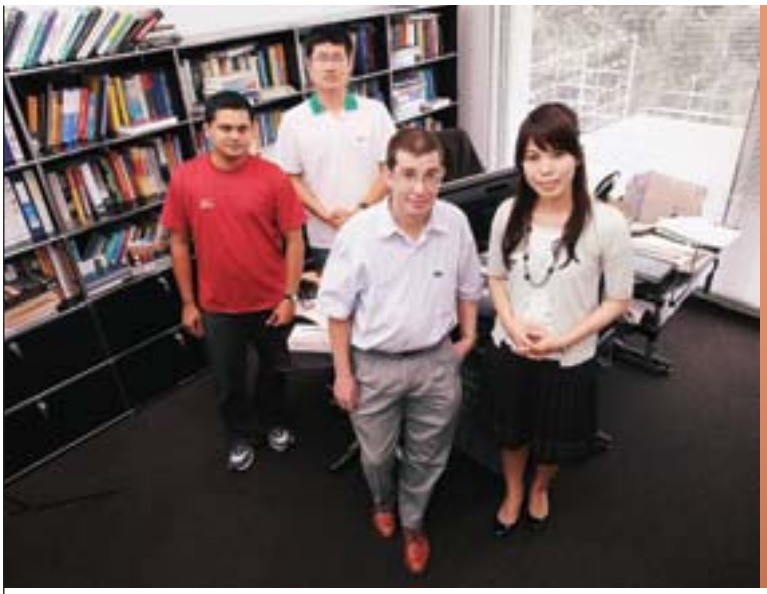
Date:17-21 November 2008
Venue: Seaside house, OIST
Co-organizers: Mary Ann Price, Ichiro Maruyama, Fadel Samatey
Speakers: Phil Beachy (Stanford Univ., USA)
Sudipto Roy (IMCB, Singapore)
Matthew Scott (Stanford Univ., USA)

James Briscoe (NIMR, London, UK)
Konrad Basler (Univ. of Zurich, Switzerland)
Caroline Hill (CRUK, London, UK)
Hitoshi Hamada (Osaka Univ., Japan)
Xi He (Harvard Medical School, USA)
Tetsuya Tabata (Univ. of Tokyo, Japan)
Wenqing Xu (Univ. of Washington, USA)
Jean-Paul Vincent (NIMR, London, UK)
John S. Parkinson (Univ. of Utah, USA)
Ikuo Kawagishi (Hosei Univ. Japan)
Ariane Briegel (Cal Tech, USA)
Victor Sourjik (Univ. of Heidelberg, Germany)
Brian Crane (Cornell Univ. USA)
Mu-ming Poo (Univ. of Calif., Berkeley, USA)
Joseph Culotti (Univ. orf Tronto, Canada)
Matthew Freeman (MRC-LMB, Cambridge, UK)
Maria Leptin (Univ. of Cologne, Germany)
Shin-ichiro Takahashi (Univ. of Tokyo, Japan)

6.2 OIST winter course: Evolution of complex systems

Date: December 8-14, 2008
Venue: Seaside house, OIST
Co-organizers: Sydney Brenner, Nori Satoh, Mary Ann Price
Speakers: Nori Satoh (OIST, Japan)
David J. Miller (James Cook Univ., Australia)
William McGinnis (Univ. of Calif., San Diego, USA)
Michael Levine (Univ. of Calif., Berkeley, USA)
Richard Harland (Univ. of Calif., Berkeley, USA)

Physics and Biology Unit



Principal Investigator:
Jonathan Miller

Research Theme:
Quantitative Comparative, Evolutionary and Biomedical Genomics.

Abstract

Comparative sequence analysis - the inference of the action of selection by comparing sequences from diverse species – has long been one of the most effective tools of modern biology. Today, its practice is based primarily on local models of uncorrelated, single-nucleotide substitution; however, our work has demonstrated that such models fail the simplest qualitative tests of consistency with whole-genome sequence data. The sequence data – in particular, "ultraconservation," a phenomenon first described by Brenner and colleagues when comparing fragments of the human and pufferfish genomes in the 1990s - can only be accounted for by linkage. The pattern of linkage exhibits a scale-invariant, or "fractal," structure. The principal consequences of this structure for current methods of comparative genomics are gross misestimates of the impact of selection on sequence conservation. Our research aims to elucidate the fractal structure and its origin, and to use it to achieve a better understanding of evolution and disease.

1 Staff

Researchers: Kun Gao, PhD (from 2008/10).
Sathish Venkatesan, PhD (from 2009/4).
Research Administrator / Secretary: Midori Tanahara

2 Partner Organizations

None

3 Activities and Findings

For over fifty years, gene sequence comparison has served as an inimitable "dry" laboratory for studying gene function and gene evolution. The basic principle was elucidated by Darwin two centuries ago: the action of selection on random variation yields adaptive evolution.

In comparative genomics, the key to interpretation of sequence data is the ability to distinguish "random variation," or neutral drift, from the action of selection. With the advent of abundant whole-genome sequence data, it has become clear that the current understanding of neutral drift as primarily driven by point mutation is grossly deficient, a failure underscored by observations of the ENCODE consortium in 2007.

In 2006, our studies revealed the contradiction between whole-genome sequences and the point mutation hypothesis, when we first demonstrated the overwhelming impact of linkage (a.k.a. 'correlations of

conservation') on genome sequence evolution. Over the last few years our extensive calculations on a variety of genomes with widely-ranging evolutionary distances have confirmed universal, homogeneous, and scale-invariant properties of this linkage that suggest it is - in large part - the outcome of neutral recombination processes whose essential features are common to most organisms. The successful implementation of comparative genomics will rely on a neutral theory of evolution that encompasses both recombination and local mutation. Such a theory will depart considerably from existing conceptions of sequence evolution as a diffusive process.

3.1 Relaxed constraints on sequence conservation lead to an algebraic distribution.

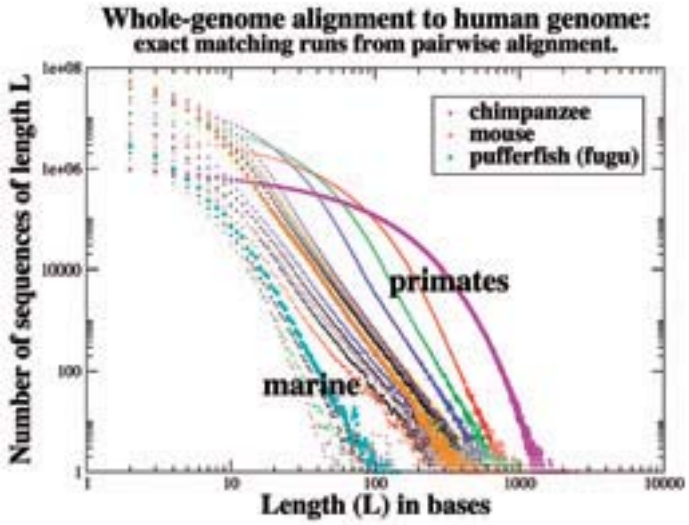


Figure3.1a: The distribution of the lengths of contiguous runs of exactly-matching sequence from pairwise whole-genome alignments (source: UCSC) of human to a variety of eukaryotes. From right to left along the bottom: chimpanzee, ape, macaque, orangutan, horse, dog, cow, cat, rabbit, guinea pig, mouse, rat, shrew, opossum, platypus, zebra finch, chicken, lizard, frog, medaka, stickleback, fugu, zebrafish, fresh-water pufferfish, lamprey, lancelet, sea urchin. On this log-log plot, chimpanzee (magenta), mouse (orange) and fugu (cyan) are highlighted. The unexpected observation is that -with the exception of alignments to organisms most recently diverged from human -over a wide range of lengths the lines are exceptionally straight, and not strongly curved.

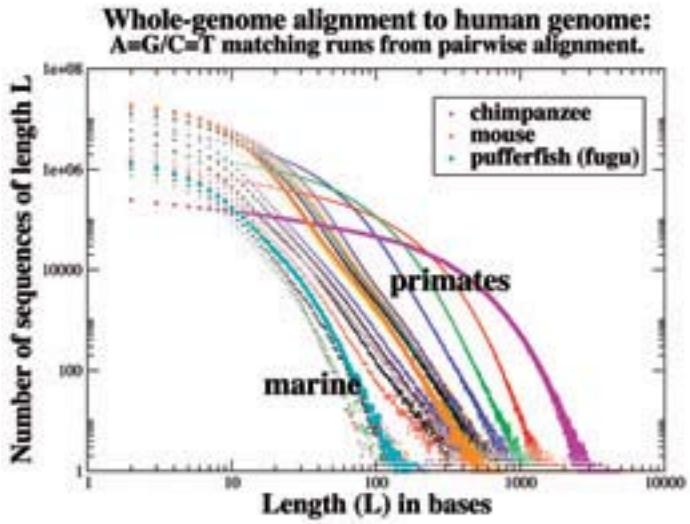


Figure3.1b: This plot is identical to figure 3.1a, with one important distinction: in computing contiguous runs of exactly-matching sequence from the pairwise whole-genome alignments, the bases 'A' and 'G' have been taken to be equivalent to one another, as have the the bases 'C' and 'T.' What is remarkable is that between figures 3.1a and 3.1b, to a good approximation all the curves have been effectively shifted to the right, which is tantamount to multiplication (or scaling) by a constant factor. The conventional wisdom about how these sequences evolved by point mutation would have suggested instead that their slopes ought to have decreased.

3.2 Repetitive sequence conservation exhibits the same algebraic distribution.

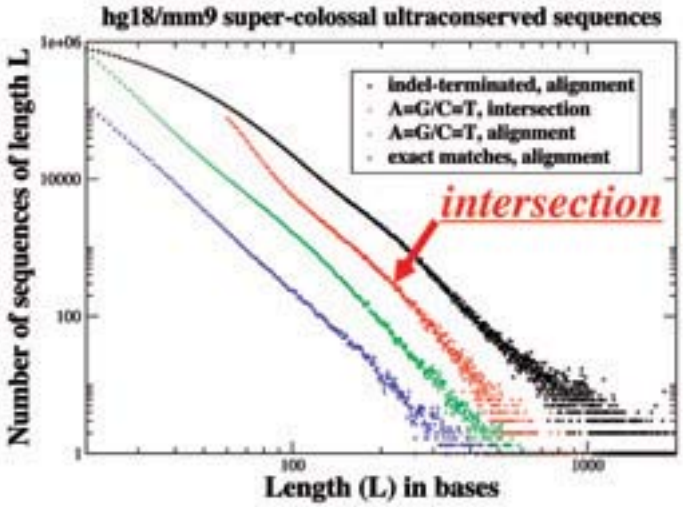


Figure 3.2: Human against mouse whole-genome intersection versus alignment. The intersection **(red)** recovers sequence absent from the corresponding alignment **(green)**. The red curve consists primarily of highly-repetitive (but single-copy) sequence. Both the red and green curves are comprised of A≡G/C≡T matching runs as in figure 3.1b. The **blue** curve displays exact matching runs as in figure 3.1a; the black curve indel-terminated runs. Both the blue curve and the **black** curve are shown solely for the sake of comparison. The overall shape of the curves for L>80 bases is largely the same. This shape and degree of similarity are shared by alignments and intersections among a diverse set of eukaryotic genomes. The similarity suggests that the mechanisms underlying the evolution of these classes of repetitive sequence and complex sequence share common features.

3.3 Recombination can be misinterpreted as selection.

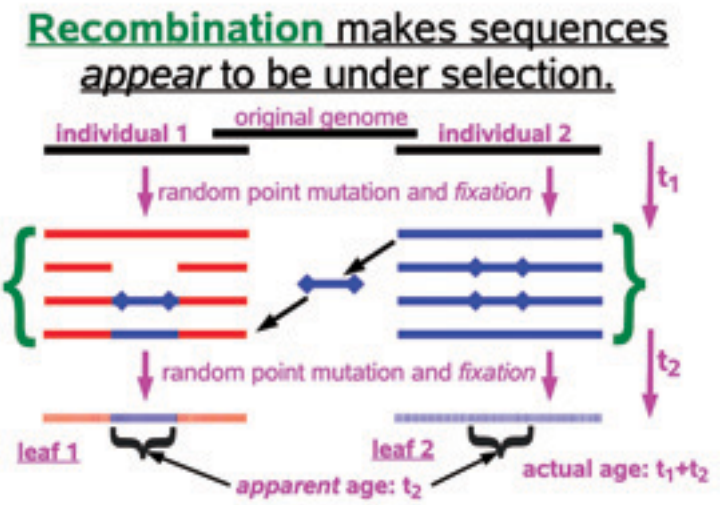


Figure 3.3: We are ordinarily able to obtain sequence data only at leaves that correspond to species not yet extinct; therefore ancestral sequences and history are only indirectly reflected in such data. This figure shows how recombination can lead to misinterpretation of a sequence's age as inferred from differences in point mutations between two leaves. Although the genomes originating from individual 1 and individual 2 have evolved by point mutation for the same periods of time, each of these genomes undergoes a different set of point mutations during this time and it is these differences that contribute to inference of the age. If we assume that a constant rate of point mutation acts -in effect -as a clock, comparison between the genomes on the two leaves makes it *appear* as if point mutations have been suppressed in the recombined segment relative to the rest of the genome. A gene-conversion event has been illustrated here, but other kinds of recombination can have a similar impact. This gene-conversion event has been chosen to remind the reader of the first step in construction of the simplest Cantor set; such recombination events over many different length scales would yield a fractal pattern.

4 Publications

4.1 Journals

Gu, P., Reid, J.G., Gao, X., Shaw, C.A., Creighton, C., Tran, P.L., Zhou, X., Drabek, R.B., Steffen, D.L., Hoang, D.M., Weiss, M.K., Naghavi, A.O., El-daye, J., Khan, M.F., Legge, G.B., Wheeler, D.A., Gibbs, R.A., Miller, J., Cooney, A.J., & Gunaratne, P.H. Novel microRNA candidates and miRNA-mRNA pairs in embryonic stem (ES) cells. *PLoS ONE* 3 (7), e2548 (2008).

Reid, J.G., Nagaraja, A.K., Lynn, F.C., Drabek, R.B., Muzny, D.M., Shaw, C.A., Weiss, M.K., Naghavi, A.O., Khan, M., Zhu, H., Tennakoon, J., Gunaratne, G.H., Corry, D.B., Miller, J., McManus, M.T., German, M.S., Gibbs, R.A., Matzuk, M.M., & Gunaratne, P.H. Mouse let-7 miRNA populations exhibit RNA editing that is constrained in the 5'-seed/ cleavage/anchor regions and stabilize predicted mmu-let-7a:mRNA duplexes. *Genome Res* 18 (10), 1571-1581 (2008).

4.2 Book(s) and other one-time publications

None

4.3 Oral presentations

Miller, J. Absence of Intrinsic scale in whole-genome comparison. OIST Junior Researcher Retreat '08, Okinawa, Japan. October 30-31, 2008.

Miller, J. Infomax_N. OIST-IRP Internal Seminars, Okinawa, Japan. December 19, 2008.

4.4 Posters

None

5 Intellectual Property Rights and Other Specific Achievements

None

6 Meetings and Events

6.1 OIST Seminar: Statistical Physics for Traffic Flow Modeling and Simulation

Date: Aug 11, 2008
Venue: IRP Conference Room
Speaker: Dr. Kun Gao, Department of Physics,
University of Science and Technology of China

6.2 OIST Seminar: The Many Masters of a Single Slave: The case of the silkworm sex determination pathway

Date Aug: 14, 2008
Venue: IRP Conference Room
Speaker: Dr. Sathish Venkatesan, Laboratory of Molecular Genetics,
Center for DNA Fingerprinting & Diagnostics

6.3 OIST Seminar: Quantum Phases of Matter

Date: Aug 19, 2008
Venue IRP Conference Room
Speaker: Dr. Dennis Dickerscheid, JSPS Postdoctoral Fellow,
Department of Physics, The University of Tokyo

6.4 OIST Seminar: A phase transition to collective behavior in eukaryotic cell populations

Date: Dec 9, 2008
Venue: Meeting Room, Seaside House
Speaker: Dr. Thomas Gregor, JSPS Post-doctoral fellow,
The University of Tokyo, Assistant Professor, Princeton University

6.5 OIST Seminar: Dynamics and precision in early embryonic development

Date: Dec 10, 2008
Venue: OITC Salon
Speaker: Dr. Thomas Gregor, JSPS Post-doctoral fellow,
The University of Tokyo, Assistant Professor, Princeton University

6.6 OIST Seminar: Microarray Data Analysis using Graph Theory: From Visualization to Outlier Detection

Date: Dec 17, 2008
Venue: IRP Conference Room
Speaker: Dr. Raymond Wan, Bioinformatics Center, Kyoto University

6.7 OIST Seminar: From Micro Scale to Macro Data in Physics and Engineering

Date: Feb 09, 2008
Venue: Seminar Room, Seaside house
Speaker: Dr. Alexander Palov, Department of Microelectronics,
Skobeltsyn Institute of Nuclear Physics, Lomonosov Moscow State University, Russia

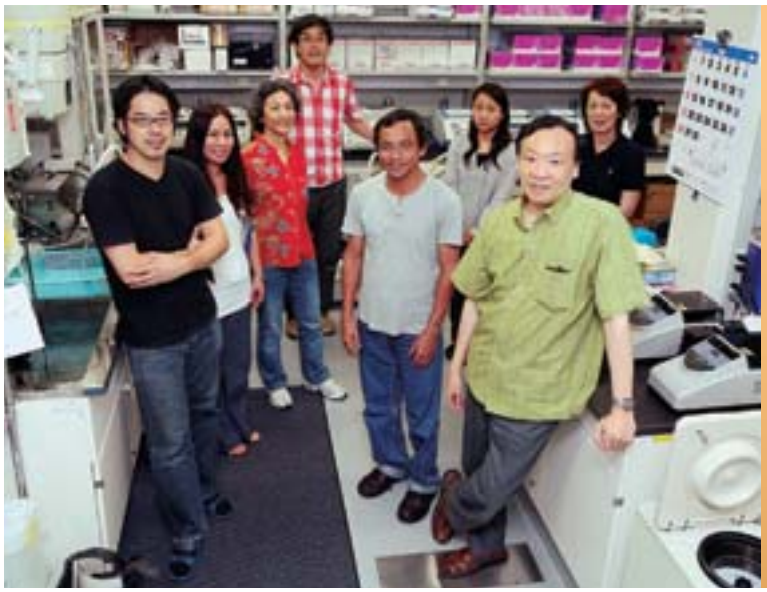
6.8 OIST Seminar: Methods of theory of dynamical systems and partial differential equations in the problem of compression of discrete signals

Date: Feb 17, 2009
Venue: Seminar Room, Seaside house
Speaker: Dr. Maxim Koroteev, Digital Media Research Institute, Samsung Electronics Ltd. Korea

6.9 OIST Seminar: Improving Constraint-Based Solvers

Date: March 09, 2009
Venue: IRP Conference Room
Speaker: Dr. Horst Samulowitz, Microsoft Research, UK

Molecular Neurobiology Unit



Principal Investigator:
Takayuki Naito

Research Theme:
Single-cell Biochemistry and Molecular Analysis of Brain Functions

Abstract

Our research aim is to provide link between genetic and molecular events occurring in cells and brain functions. The major themes of our research are 1) analysis of activity-dependent gene expression, 2) classification of neuronal cell types based on gene expression (the Neumap project), and 3) identification of unknown signal molecules in intercellular fluid in the brain. In addition, we are establishing molecular neuroscience of salamander having much larger cells compared to mammalian.

Gene expression analysis is the basic technology to carry out the research. We carried out the gene expression analysis of small regions of mouse brain and the analysis of single cell type (mouse Purkinje cell) in this fiscal year and the results are summarized in the following section. Now we are investigating single-cell analysis. We are collecting data on activity-dependent gene expression using primary culture of mouse hippocampal cells, cerebellar granule cells, and hippocampal slices (not described in this report).

To study dynamics of neuropeptides in the living brain, we developed a new type of microdialysis-based apparatus for in vivo peptide collection followed by analysis using a highly sensitive mass spectrometer.

In 2006, we have started salamander project to establish molecular neuroscience of salamander, because salamander has large cells and is thought to be a good material for imaging studies, electrophysiology, and single-cell analysis of the nervous system. In this fiscal year, we completed full-length sequencing of about 16,000 cDNAs from brain, retina, and spinal cord of salamander and we are constructing a cDNA database of them. We made a brain atlas of salamander and carried out axonal tract-tracing using fluorescent dyes to elucidate the connectivity of neurons. These results provide fundamental information for neuroscience of salamander.

1 Staff

Researchers: Kiyotaka Akiyama
Setsuko Nakanishi
Nozomu Nakamura
Michael Chandro Roy
Technical Staff: Seiko Kuraba
Sayaka Arai
Saori Ishida
Research Administrator / Secretary: Kaori Yamashiro

2 Partner Organizations

Okinawa Institute of Science and Technology, PC
Type of partnership: Joint research
Name of principal researcher: Dr. Sydney Brenner
Research theme: Salamander project

University of Tokyo, Department of Metabolome, Graduate School of Medicine
Type of partnership: Joint research
Name of principal researcher: Prof. Ryo Taguchi
Research theme: Lipids in the nervous system

Hamamatsu University School of Medicine
Type of partnership: Joint research
Name of principal researcher: Prof. Mitsutoshi Setou
Research theme: Mass Imaging

University of Kuopio, Finland
Type of partnership: Joint research
Name of principal researcher: Dr. Kazuhiko Tatemoto
Research theme: Peptidome in the brain

Keio University, Department of Biosciences and Informatics
Type of partnership: Joint research
Name of principal researcher: Prof. Daisuke Uemura
Research theme: Marine microorganism project

Osaka Bioscience Institute
Type of partnership: Joint research
Name of principal researcher: Dr. Shigetada Nakanishi
Research theme: Study of cerebellum

3 Activities and Findings

3.1 Gene expression analysis

Neural cells in the brain are classified into highly diverse cell types. For understanding of neuronal system, it is important to know precise gene expression in each of diverse cell types and very small regions of the brain. We analyzed gene expression of one cell type and seven regions of mouse brain.

3.1.1. Purkinje cell

For construction of gene expression profile of Purkinje cells, we collected Purkinje cells by laser capture microdissection (LCM) and isolated RNAs from the Purkinje cells. Following amplification of mRNA, genome-wide expression profile was constructed using microarray analysis. We selected 377 genes (which are classified into neurotransmitter synthesis enzyme, neuropeotide, ligand-gated channel, and G-protein coupled receptor) that play important roles on neuronal activities. We also applied Real-Time PCR method for these genes.

We used Affymetrix gene chip for the construction of genome-wide expression profile. We prepared two sets of fluorescent labeled RNAs using two-cycle amplification system initially from two different materials for checking reproducibility of the analysis and hybridized them to microarrays (Affymetrix GeneChip 430 2.0.). Since many probe-sets on the microarrays were misdesigned and could not hybridize to their target RNA, we selected 26,386 probe-sets whose sequence accuracy was validated. Among 26,386 probe-sets, 12,736 and 13,057 probe-sets had Present call (P call) in respective hybridization and 11,349 probe sets had P call in both samples. Thus, reproducibility of P call was 89.1% (11,349/12,736) and 86.9% (11,349/13,057). Coefficient of determination (R^2) of signal values between two samples was 0.991. Therefore, we consider that two hybridizations have reliable data.

In order to know their accurate mRNA amount, we examined the expression of 377 selected genes by real-time PCR method. Real-Time PCR was carried out using cDNA, synthesized from RNAs amplified by T7 polymerase. We used mouse genomic DNA as standard because the number of each gene that exists in a single cell is mostly two and weight of genomic DNA per single cell is known to be 2.5 pg. In Table 1, the results of Affymetirix Gene Chip and Real-Time PCR analyses are summarized. Among the examined 377 genes, mRNAs of 60 and 65 genes were detected by Real-Time PCR and gene chip analyses, respectively. Both methods detected mRNAs of 36 genes.

Table 1. Number of genes whose mRNAs were detected by two methods

Category	Number of selected Gene	Number of the genes whose mRNAs were detected	
		Real Time PCR[1)]	Gene Chip[2)]
Neurotransmitter Synthesis enzyme	34	13 (26)	16
Neuropeptide	62	9 (36)	10
Ligand-gated channel	61	19 (37)	20
G- protein coupled receptor(GPCR)	220	19 (31)	19
Total	377	60(130)	65

1) The Number of genes whose mRNAs detected to be over one copy per cell. Parenthesis indicated the number of genes whose cDNA were detected in 0.25 ng cDNA.
2) Number of genes showed Present or Marginal call.

For example, seven neuropeptide genes with high expression level are shown in Table 2. Cck(cholesystokinin) gene is highly expressed (over 50% of all neuropeptide mRNAs). This high expression of Cck gene might indicate an important unknown role of Cck in Purkinje cells. This gene expression profiling might help us to understand the molecular mechanism about the function of Purkinje cells in response to synaptic stimulation.

Table 2. Expression of Neuropeptide genes

Symbol	Gene Name	Expression	
		Real-time PCR[1)] (%)	Affymetrix[2)]
Cck	cholecystokinin	53.5	603
Penk1	preproenkephalin 1	12.4	122
Npy	neuropeptide Y	9.0	94
Agt	angiotensinogen	8.1	86
Adcyap1	adenylate cyclase activating polypeptide 1	8.1	35
Tshb	thyroid stimulating hormone, beta subunit	2.9	61
Gal	galanin	1.8	A[3)]

1) The number shows the ratio of total transcripts of 62 neuropeptide genes.
2) The value is fluorescent intensity of each probe-set of Affymetrix gene chip.
3) "A" indicates absent call.

3.1.2. cerebellar cortex

Anatomical and physiological studies of mouse cerebellum have been well studied. However, precise gene expression of each cell type in cerebellum remains to be studied. We examined gene expression of four regions of cerebellar cortex in which cell types and neural pathway have been well described. These regions were microdissected by LCM, and we quantified the copy number of mRNA coding for 33 neurotransmitter synthetic enzymes (acetylcholine, adrenaline, dopamine, GABA, histamine, melatonin, nitric oxide, noradrenalin, serotonin) per nanogram of cDNA using real-time PCR. In addition to GABA synthetic enzymes, we detected the mRNAs of histamine(hisitidine decarboxylase), serotonin(tryptophan hydroxylase 2), glycine(hydroxymetyltransferase 1), and L-Dopa (serine and tyrosine hydroxylase) synthetic enzymes in Purkinje cell layer. Because Purkinje cell is known to be GABAergic neuron, this observation is interesting to follow up research. Since Purkinje cell layer contains afferent fibers and Glia cell's spines whose cell bodies locate on other regions, further study is needed to identify cells which really express these mRNAs.

3.1.3. Hippocampal subregional differences of gene expression in ligand-receptor system

The hippocampus playing an important role in spatial and episodic memories contains three main subregions, CA1, CA3, and dentate gyrus (DG). These three regions have different modalities of input information through the ligand-receptor systems for excitatory glutamatergic, inhibitory GABAergic, cholinergic, serotoninergic, neuropeptidergic, etc. In the excitatory glutamatergic system, information flows from entorhinal cortex (EC) superficial layer (layer II) to DG to CA3 and finally to CA1, referred to as the trisynaptic pathway. CA1 also contains a parallel excitatory monosynaptic pathway from EC layer III. CA3 neurons have robust autoassociational connections to neighboring CA3 neurons. Moreover, other systems differentially contribute to CA1, CA3, and DG, depending on ligand-receptor relationships. Thus, each hippocampal subregion is proposed to differentially integrate input information and establish different regulation systems. However, the differences of the ligand-receptor systems among CA1, CA3, and DG remain unclear. To determine the difference in the hippocampal subregions, we have demonstrated gene expression patterns and copy numbers per nanogram of cDNAs in classical neurotransmitter-dependent synthesizing enzymes (34 genes), neuropeptides (64 genes), ligand-gated channels (62 genes), and G protein-coupled receptors (GPCRs, 202 genes).

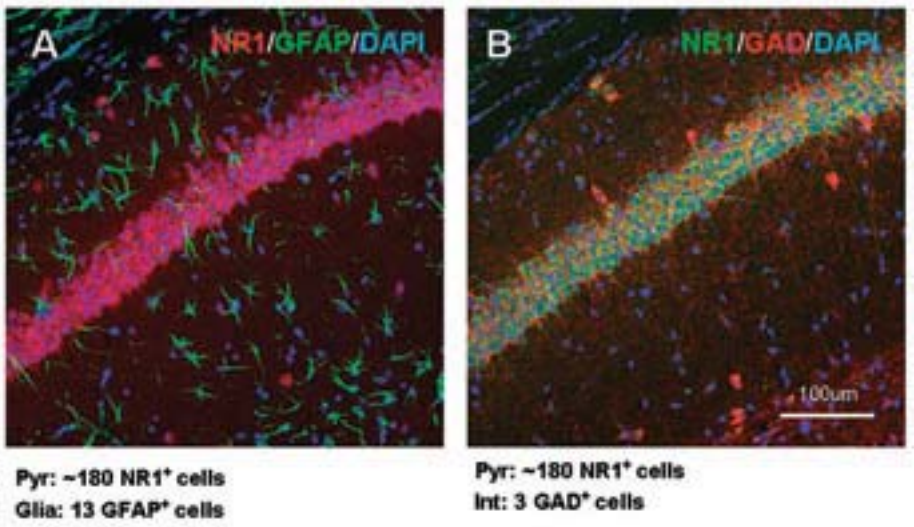


Fig. 1 Cell population in CA1 pyramidal cell layers

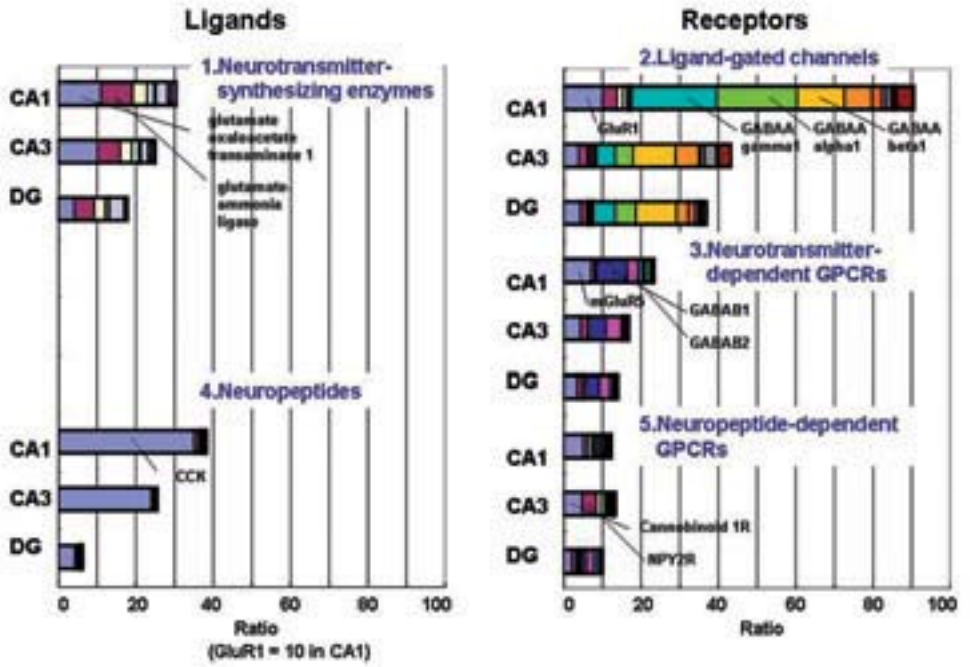


Fig.2 Population of gene expression

Using immunohistochemistry, we found 90% of NR1 positive cells and 10% of GAD positive and GFAP positive cells in these cell layers (Fig.1), supporting that each cell layer contains 90% of hippocampal principal neurons. Then, we dissected and collected CA1 and CA3 pyramidal cell layers and DG granule cell layers from adult mouse hippocampal sections using Laser capture microdissection. Using these samples of each cell layer, we employed real-time RT-PCR. Our results showed different expression patterns of the glutamate-GABA systems in CA1, CA3, and DG (Fig.2). CA1 expressed abundant GABA receptors (*GABAA receptor gamma I, GABAA receptor alpha 1, and GABAB1 receptor*), and abundant glutamate receptors (*GluR1* and *mGluR5*) compared to CA3 and DG. CA1 and CA3 predominantly expressed *Cck,* but DG did not express. Furthermore, in these three subregions, a variety of neuropeptide-dependent GPCRs (*cannabinoid 1 receptor, neuropeptide Y2 receptor, neurotensin 2 receptor, opioid receptor-like 1, and somatostatin 3 receptor*) was expressed in different patterns and copy numbers (Fig.2).

In the classical neurotransmitter and neuropeptide systems, we propose that hippocampal CA1, CA3, and DG have different multimodalities of input information. These results provide fundamental evidence to understand the functional differences of hippocampal CA1, CA3 and DG.

3.2 Mass spectrometric analysis of biomolecules in the nervous system

3.2.1. Method development for the collection of endogenous peptide from living animal brain

Neuropeptides play a significant role in the function of brain and animal behavior. To understand their full function and specific location in the brain, it is important to identify them quantitatively and study their dynamics in vivo. Microdialysis (MD) is a widely used research tool for sampling molecules of interest (such as neurotransmitters, drugs) from living animals. However, a quantitative recovery of neuropeptides is still a draw back. There is a scope for further improvement of MD for neuropeptides sampling. We are developing an improved MD-based micro-probe for high recovery of peptides in vivo. The new probe showed a quantitative in vitro recovery of peptides (70-90% at 0.05-1 μl/min perfusion flow rate). This micro-probe will be used for sampling of peptides from a freely moving animal brain, and the collected peptides will be analyzed by a high sensitive NanoLCMS (Nano-Liquid Chromatography Mass) system that we developed already.

3.2.2. Imaging mass spectrometry to visualize phospholipids in tissue section

It is important to study specific location of bioactive molecules to understand their functions. Imaging mass spectrometry system (IMS) is one of good technologies for the purpose. In collaboration with Dr. Setou and Dr. Taguchi groups, we visualized and identified phospholipids in a mouse retina section (8 μm) by IMS. In the spectrum obtained from a raster scan, sixteen ion signals with high intensity were selected and analyzed with MS/MS. These signals were annotated from four phosphotidylcholine molecules. These phospholipids were identified as PC (16:0/16:0), PC (16:0/18:1), PC (16:0/22:6), and PC (18:0/22:6). A reconstructed ion images of the mouse retina showed that PC (16:0/18:1) distributed in the optic fiber layer, the ganglion cell layer, and the inner plexiform layer. Whereas, PC (16:0/16:0) distributed in the inner nuclear layer and the outer plexiform layer and PC (18:0/22:6) distributed in the outer and inner segments of the visual cell layer. These PC molecules clearly form three layers in the retina. The distinguished layers of PCs in the retina suggest that they might have specific function. However, the function of fatty acids in the retina is unknown. We speculate that PC (18:0/22:6) with an unsaturated fatty acid DHA (22:6) might play a key role in phototransduction mechanisms in the visual cells by increasing membrane fluidity and enhancing conformational change of rhodopsin to metarhodopsin.

Using this imaging mass spectrometry system, we are currently analyzing salamander retina. Since salamander has large cell size compared to other vertebrates, we are expecting to visualize cellular level phospholipids distribution in the salamander retina.

3.3 Salamander project

The salamander is thought to be a good material to study the nervous system because of large cell size and relatively simple nervous system. We compared the cell size, genome size and mRNA content per cell between the salamander and the mouse and found that these sizes and mRNA content of salamander are over 10-fold higher than those of the mouse. We selected *Ambystoma*

mexicanum as our material. The retina of *A. mexicanum* has the same structural organization with murine retina but the cell size is larger than that of mouse (Fig. 3). We have constructed brain atlas of *A. mexicanum* by using coronal, horizontal, and sagittal sections stained with Klüver-Barrera method and Bodian's method (see annual report 2007). The brain of *A. mexicanum* is subdivided into three parts, e.g., forebrain, midbrain and hindbrain, which have the fundamentally the same organization with the mammalian brain (Fig. 4). To elucidate the connectivity between different populations of neurons, axonal tract-tracing experiment was done by iontophoretic microinjection of biocytin and visualized with fluorescent dyes. In *A. mexicanum* the habenula nucleus is readily accessible compared to that of rodents. We iontophoretically injected biocytin into the dorsal habenular nucleus and demonstrated anterogradely labeled cell bodies of ventral habenula nucleus and retrogradely labeled axon bundles, the fasciculus retroflexus, projected to the interpeduncular nucleus (Fig. 5). We are constructing a three-dimensional brain atlas using 277 serial sections (data not shown).

We have constructed cDNA libraries of brain, retina, and spinal cord of *A. mexicanum.* We completed first-run sequencing of 5'-ends of 100,000 clones from the brain library, 50,000 clones from the retina library, and 50,000 clones from the spinal cord library. These sequences were assembled into 16,195 clusters, which is almost equivalent to the number of cloned genes. We carried out full-length sequencing of the representative clones from each cluster. We are now constructing a cDNA database of them.

We selected 737 genes from mouse genome which are thought to play important roles in neuronal function and analyzed their orthologs in salamander genes by BLAST (Basic Local Alignment Search Tool) analysis. We identified 165 orthologs from the above analysis, which were categorized into 19 neurotransmitter synthetic enzymes, 20 neuropeptides, 68 G-protein coupled receptors, 29 ligand-gated channels and 29 voltage-gated channels.

These results are fundamental information for neuroscience of salamander.

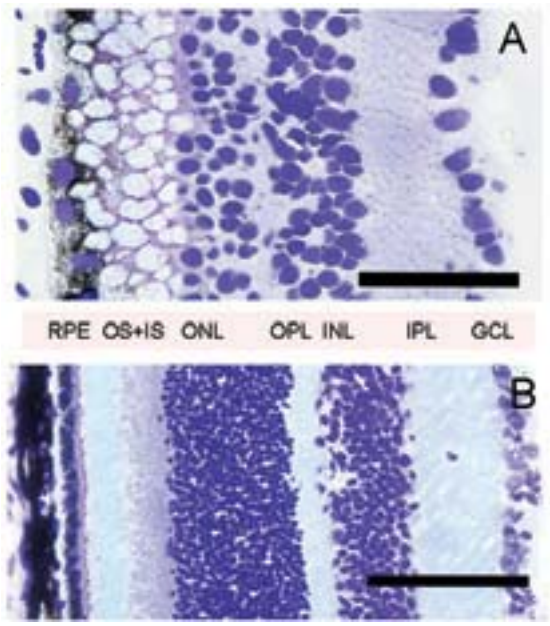


Figure3: Retina of *Ambystoma mexicanum* (A) and *Mus musculus* (B)
The retina of *A. mexicanum* and *M. musculus* has similar organization of layers, however, *A. mexicanum's* retina has large-size cell and a small number of cell compared to those of *M. musculus*. Abbreviations; RPE: retinal pigment epithelium, OS: outer segments, IS: inner segments, ONL: outer nuclear layer, OPL: outer plexiform layer, INL: inner nuclear layer, IPL: inner plexiform layer, GCL: ganglion cell layer. Bar: 100 μm.

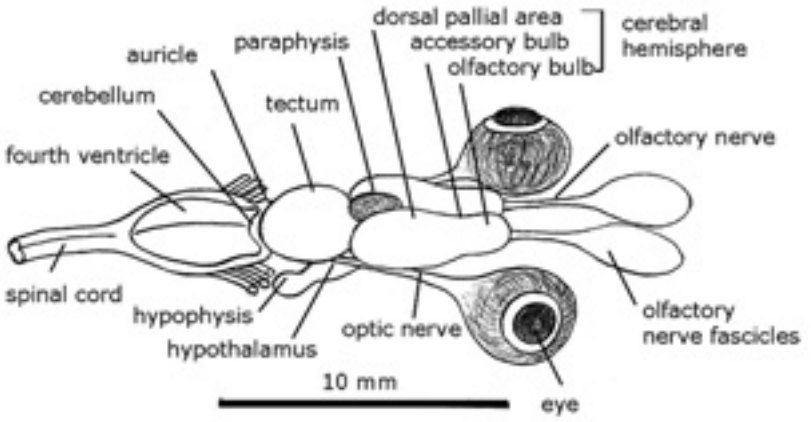


Figure4: Structure of the nervous system of *Ambystoma mexicanum*

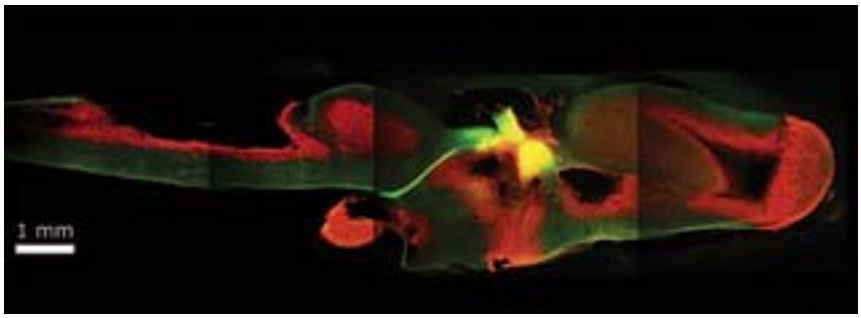


Figure5: Neuroanatomical tract-tracing in the brain of *Ambystoma mexicanum*
Biocytin injected iontophoretically into the dorsal habenular nucleus was retrogradely transported into ventral habenular nucleus. At the same time, biocytin was anterogradely transported toward the interpeduncular nucleus, showing the fasciculus retroflexus.

4 Publications

4.1 Journals

Akiyama, K., Nakanishi, S., Nakamura, N.H., & Naito, T. Gene expression profiling of neuropeptides in mouse cerebellum, hippocampus, and retina. *Nutrition* 24 (9), 918-923 (2008).

Hayasaka, T., Goto-Inoue, N., Sugiura, Y., Zaima, N., Nakanishi, H., Ohishi, K., Nakanishi, S., Naito, T., Taguchi, R., & Setou, M. Matrix-assisted laser desorption/ionization quadrupole ion trap time-of-flight (MALDI-QIT-TOF)-based imaging mass spectrometry reveals a layered distribution of phospholipid molecular species in the mouse retina. *Rapid Commun Mass Spectrom* 22 (21), 3415-3426 (2008).

4.2 Book(s) and other one-time publications

Nothing to report.

4.3 Oral presentations

Naito, T., Akiyama, K., Nakanishi, S., Brenner, S. Towards neuroscience of salamander, The 79th Annual Meeting of the Zoological Society of Japan, Fukuoka university, Fukuoka, Japan, September 5 - 7, 2008

Brenner, S., Akiyama, K., Naito, T. Towards neuroscience of salamander (I) ---cDNA analysis of Salamander---, BMB (Biochemistry and Molecular Biology) 2008, Port Island Kobe, Kobe, Japan, December 9-12, 2008

Akiyama, K., Nakanishi, S., Naito, T. Gene expression analysis of single type neuron ---Mouse cerebellar Purkinje cells---, BMB (Biochemistry and Molecular Biology) 2008, Port Island Kobe, Kobe, Japan, December 9-12, 2008

4.4 Posters

Akiyama, K., Nakanishi, S., Naito, T. Determination of Cell Number of Ambystoma mexicanum Retina, The 79th Annual Meeting of the Zoological Society of Japan, Fukuoka university, Fukuoka, Japan, September 5 - 7, 2008

Nakanishi, S., Akiyama, K., Naito, T. Histochemical Determination of Cell types of Ambystoma mexicanum Pituitary Gland, The 79th Annual Meeting of the Zoological Society of Japan, Fukuoka university, Fukuoka, Japan, September 5 - 7, 2008

Akiyama, K., Nakanishi, S., Naito, T. Gene expression analysis of single type neuron ---Mouse cerebellar Purkinje cells---, BMB (Biochemistry and Molecular Biology) 2008, Port Island Kobe, Kobe, Japan, December 9-12, 2008

Nakamura, N., Akiyama, K., Nakanishi, S., Naito, T. Gene expression analysis of ligand-receptor system of mouse hippocampus, BMB (Biochemistry and Molecular Biology) 2008, Port Island Kobe, Kobe, Japan, December 9-12, 2008
Nakanishi, S., Akiyama, K., Naito, T. Gene expression analysis of neurotransmitter synthetases in each of the layers of mouse cerebellum, BMB (Biochemistry and Molecular Biology) 2008, Port Island Kobe, Kobe, Japan, December 9-12, 2008

Brenner, S., Akiyama, K., Naito, T. Towards neuroscience of salamander (I) ---cDNA analysis of Salamander--- , BMB (Biochemistry and Molecular Biology) 2008, Port Island Kobe, Kobe, Japan, December 9-12, 2008

Naito, T., Nakanishi, S., Akiyama, K., Brenner, S. Towards neuroscience of salamander (II) ---Neuronal research of salamander---, BMB (Biochemistry and Molecular Biology) 2008, Port Island Kobe, Kobe, Japan, December 9-12, 2008

Nakanishi, H., Nakanishi, S., Naito, T., Tajima, Y., Taguchi R. Analysis of lipid distribution of mouse retina by laser capture microdissection and MS spectrometry, BMB (Biochemistry and Molecular Biology) 2008, Port Island Kobe, Kobe, Japan, December 9-12, 2008

Tajima, Y., Nakanishi, H., Akiyama, K., Naito, T., Taguchi, R. Analysis of lipid distribution of mouse cerebellum by MS spectrometry, BMB (Biochemistry and Molecular Biology) 2008, Port Island Kobe, Kobe, Japan, December 9-12, 2008

5 Intellectual Property Rights and Other Specific Achievements

Nothing to report.

6 Meetings and Events

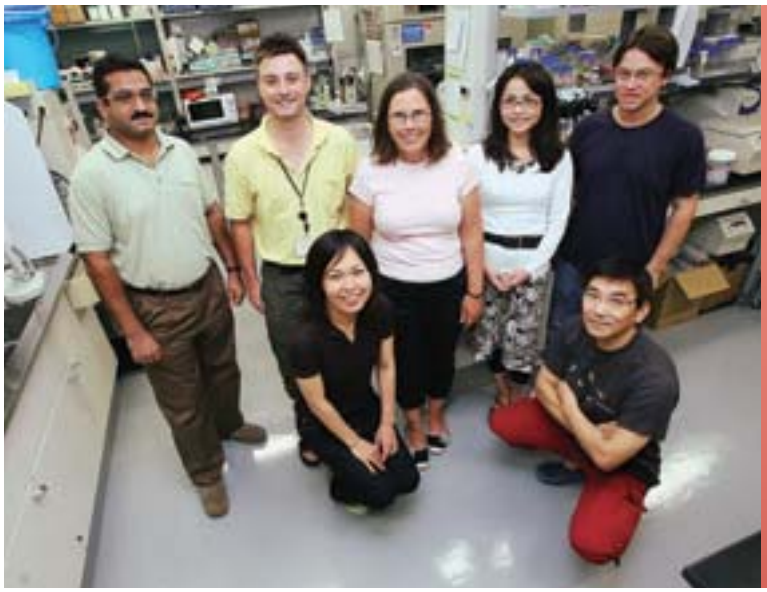
6.1 Genes and the Brain, Lecture at Kagoshima University

Date: May 26, 2008
Venue: Kagoshima University, Faculty of Medicine, Kagoshima, Japan
Speaker: Takayuki Naito

6.2 Molecular Analysis of Brain Functions, Lecture at Kyoto University

Date: July 10, 2008
Venue: Kyoto University, Department of Biophysics, Kyoto, Japan
Speaker: Takayuki Naito

Developmental Signalling Unit



Principal Investigator:
Mary Ann Price

Research Theme:
Genetic, biochemical, and cell biological studies on the mechanism of Hedgehog signalling

Abstract

The goal of our research is to understand the mechanism of the developmentally and medically important Hedgehog (Hh) signal transduction pathway, that is, how the extracellular Hh ligand brings about changes (primarily in gene expression) in its target cells. To this end, we use a variety of approaches, including *Drosophila* molecular genetics, biochemistry, cell biology, and functional genomics. Currently, our projects are focused on

- identifying novel binding partners for Hh signalling components, such as the transcription factor Cubitus interruptus (Ci), because many of these proteins are likely to be regulators of pathway activity
- understanding the subcellular localization of Hh signalling components, since this is known to be regulated on multiple levels by Hh signalling
- elucidating the mechanism of partial degradation of Ci by the proteasome, including an exploration of the evolutionary conservation of Ci processing in primitive metazoans
- using functional genomic approaches (*e.g.*, genome-wide RNAi screens in cultured cells) to identify novel Hh pathway components

1 Staff

Researchers: Dr. Chiemi Spratt
Dr. Ingo Lehmann
Dr. Yawer Hussein
Dr. Spencer Spratt
Technical Staff: Dr. Jun Isoe
Dr. Satoshi Hasegawa
Ms. Michiko Arai
Research Assistant / Graduate Student: Dr. Yifei Wang (University of Sheffield)
Research Administrator / Secretary: Ms. Chika Azama

2 Partner Organizations

3 Activities and Findings

The Hh family of extracellular signalling molecules regulates many important events during the development of animals from *Drosophila* to human. In *Drosophila*, where it was first discovered, Hh is involved in many processes including the anterior-posterior patterning of embryonic segments and larval imaginal discs (precursors of adult appendages) and the regulation of ovarian somatic stem cells. In vertebrates, the Hh family members pattern tissues such as the limb and neural tube and are involved in the development of many organs. More recently, Hh signalling has been postulated to be involved in the organization of the body plan in basal metazoa such as sea anemone, suggesting that the pathway has

ancient evolutionary roots in the animal kingdom. Mutation of components of the Hh signalling pathway in humans leads to congenital developmental disorders, such as holoprosencephaly and polydactyly, while misregulation of the pathway later in life can lead to initiation and/or maintenance of many types of cancer.

Hedgehog signalling causes changes in target cells and tissues primarily by regulating gene transcription. In the absence of Hh signalling, the pathway transcription factor (Cubitus interruptus (Ci) *in Drosophila*) is processed by limited proteolysis to a shorter protein (Ci-75) that represses transcription. Hh signalling blocks this processing and converts full-length Ci (Ci-155) to a more potent transcriptional activator, in part by stimulating nuclear localization of Ci-155. My lab has been focused on the regulation of Ci, primarily its processing to Ci-75.

During the FY2008, one new post-doc joined the lab: Dr. Spencer Spratt. One technician (Dr. Jun Isoe) left the lab and two (Dr. Satoshi Hasegawa and Miss Michiko Arai) joined. Dr. Yifei Wang successfully defended his Ph.D. thesis in November 2008 in the University of Sheffield, Sheffield, UK, and visited OIST for a short time in summer 2008. Our aims remain largely the same as reported in the FY2007 annual report. To avoid repetition, I have only briefly introduced each topic here; please see that report for further information.

Project 1: Identification and characterization of Ci binding partners.

Previously, Dr. Alexander Soloviev used tandem affinity purification (TAP) followed by mass spectrometry (MS) to identify novel binding partners for Ci. Drs. Ingo Lehmann and Chiemi Spratt have continued trying to validate these potential Ci binding partners by confirming their interaction with Ci and by determining if and how they function in Hh signalling and/or Ci regulation. Dr. Lehmann successfully reproduced Ci binding assays for Cos2 (a protein already known to bind Ci), but was unable to confirm than any of our novel "hits" interact with Ci (Figure 1). His data suggest that either our "hits" are artifacts or that their binding to Ci is so weak as to be undetectable by methods less sensitive than MS. Since at least one of our "hits" has an effect on Hh-mediated transcription, we think the latter explanation is true in at least some cases, and we continue to try to understand how this particular protein affects Hh signalling (see Project 2 below, for example).

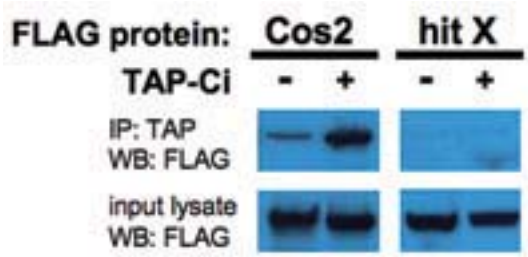


Figure 1. Results of TAP IP Western blot (WB) assays. Cos2 co-IPs with Ci, however a protein identified by MS as a Ci interactor (hit X) does not.

Dr. Lehmann has continued to develop the TAP-MS approach that Dr. Soloviev initiated in the lab. He is attempting to use new TAP tags to improve yield and has recently secured funding from JSPS to extend our studies of Ci/Gli binding proteins to vertebrate cells.

Project 2: Subcellular localization of Hh signalling components.

In vivo, full-length Ci only enters the nucleus when cells receive the Hh signal. Both Drs. Chiemi Spratt and Ingo Lehmann found that our attempts to study Ci nuclear localization using exogenously expressed tagged Ci in cultured fly cells (by immunofluorescence and by biochemical separation, respectively) forces some Ci into the nucleus, even in the absence of Hh, and thus is not a valid model system for studying Ci localization (Figure 2). Dr. Yawer Hussein is developing assays and reagents for studying Ci localization *in vivo* in *Drosophila* wing imaginal discs. Once he establishes these assays in our lab, he will use them to determine whether the novel "hits" from TAP-MS experiments above affect Ci localization using inducible RNA interference (RNAi) lines.

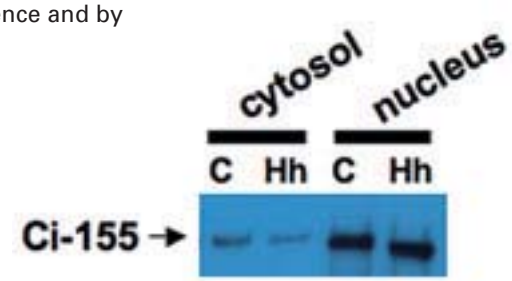


Figure 2. Biochemically fractionated Kc cells expressing Ci, - or + Hh, as designated. Even in the absence of Hh, a large portion of Ci is nuclear (compare lane 3 to lane 1).

Project 3: Mechanism of Ci partial proteolysis by the proteasome and its evolutionary conservation.

Previously, Yifei Wang did a Ci deletion and mutational analysis to determine which regions of Ci are required for Ci-75 formation (described in the 2007 annual report and see 4.1 (Publications) below). Briefly, we showed that the Ci zinc finger region and a particular lysine residue (K750) together form a protection signal that prevents the complete degradation of Ci by the proteasome. Furthermore, the zinc fingers, the C-terminal domain, and the PKA-, GSK3-, and CK1-phosphorylated region are each required for initiation of proteasomal degradation. To continue with this project, our focus has shifted to two questions about the conservation of the mechanism of Ci processing: (1) are the domains we identified as being important for Ci processing also important for vertebrate Gli3 processing and (2) given the conservation of the Hh pathway in basal metazoa, is Ci processing also conserved in these organisms? To approach the first question, we are studying the processing of human Gli3, wild-type and various mutants, in *Drosophila* and vertebrate cells. All the Ci regions important for processing are conserved in human Gli3, with the possible exception of K750 (it is difficult to identify an exact counterpart of a single residue given the overall low conservation between Ci and Gli3). In order to gain expertise in studying Hh signalling in vertebrate cells, I spent several months in FY2008 working in the lab of Dr. James Briscoe (NIMR, London, UK); however, our results are too preliminary to present at this time. To answer the second question we are studying the processing of Ci from basal metazoa in cultured fly cells and in wing discs, since carrying out experiments in these organisms is difficult or impossible at the present time. I have established a collaboration with Dr. David Miller, one of the lecturers at the OIST Winter Course on Evolution of Complex Systems, to study coral Ci. The coral genome sequence is as yet unpublished, but we have obtained the coral Ci cDNA from Dr. Miller to begin our studies in *Drosophila*. In the meantime, a comparison of the *Drosophila* Ci sequence with Ci from limpet (Lottia gigantea) and a coral relative, sea anemone (Nematostella vectensis) (two recently sequenced genomes on the JGI public database), shows that most of the regions important for processing (the zinc fingers, the phosphorylated region, and the C-terminus) are conserved (Figure 3), though sea anemone (and coral) contain a short C-terminal extension.

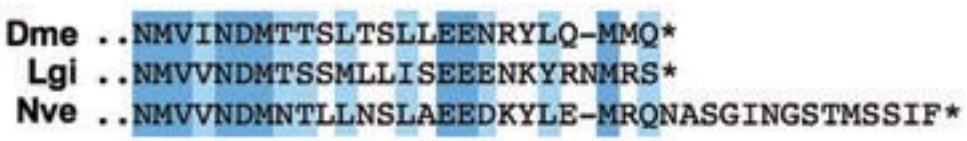


Figure 3. Aligment of the C-terminus of Ci from Drosophila melanogaster (Dme), Lottia gigantea (Lgi), and Nematostella vectensis (Nve).

Project 4: Genome wide RNAi screen to identify novel regulators of Ci proteolysis.

Previously, Yifei Wang developed a high throughput assay for Ci proteolysis that involves stably expressing a Firefly-Ci-Renilla luciferase fusion protein in cultured fly cells and quantifying the Renilla/Firefly luciferase ratio as a measure of Ci processing. In FY2007 he screened the entire fly genome for genes required in Ci processing using the collection of double-stranded RNAs (dsRNAs) at the *Drosophila* RNAi Screening Center in Harvard, USA, and found that ~240 genes had an effect on Ci proteolysis. Drs. Satoshi Hasegawa and Spencer Spratt, with help from Miss Michiko Arai, have been following up Dr. Wang's work using the following secondary screens (1) repeating the RNAi assay on cells expressing the Firefly-Ci-Renilla reporter using the dsRNA used in the screen and a second non-overlapping dsRNA, (2) conducting an immunoprecipitation (IP)-Western Blot (WB) assay on RNAi treated cells to determine directly whether Ci-75 is formed, and (3) determining whether Ci processing is affected *in vivo* in the wing imaginal disc of third instar larvae using inducible RNAi lines.

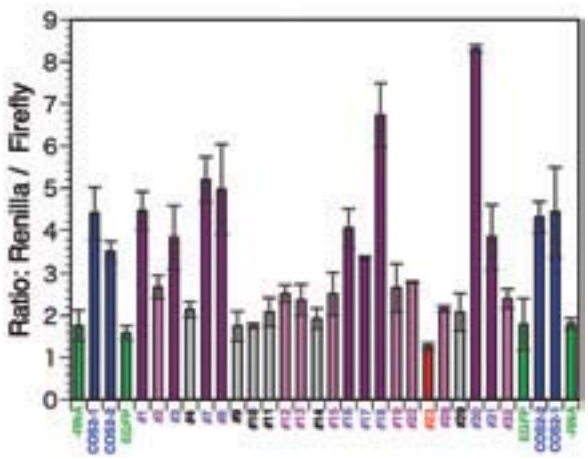


Figure 4. A bar chart showing the Renilla/ Firefly luciferase ratio for negative controls (green), cos2, a positive control (blue), strong hits which block Ci processing (dark purple), weaker hits which block Ci processing (light purple), hits which enhance Ci processing (red), and hits having no effect (gray).

Figure 5. Results of IP-WB, showing that RNAi B-F have no effect on Ci-75 formation, but that RNAi A blocks Ci-75 formation. A is a proteasome component. The star marks a background band.

Dr. Hasegawa has re-tested about 145 of Yifei's original hits and finds that about 30% (45/145) re-test positively in the secondary screen (Figure 4). He has had some difficulty getting the IP-WB assay working consistently, though for an example, see Figure 5.

In the wing imaginal disc, Ci is expressed in the anterior (A) compartment, while Hh is expressed in the posterior (P) compartment. Hh diffuses to cells in the A compartment, near the A/P border, blocking Ci processing and leading to higher levels of full-length Ci in a stripe along the A/P border. Clones of cells lacking components required for Ci processing located in the A compartment even at a distance from the A/P border will express high levels of full-length Ci. Dr. Spratt has used this assay to test ~30 RNAi lines corresponding to our "hits". So far the only genes to test positively in this assay are ones already known to be involved in Ci processing (Figure 6), but these results are promising and demonstrate that the assay is clearly working well.

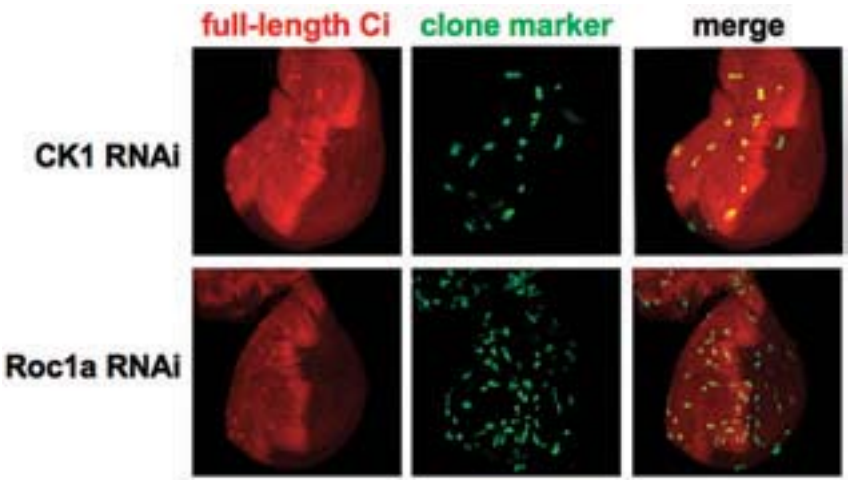


Figure 6. Clones (marked with GFP in green) expressing RNAi for CK1 (upper panel) and Roc1a (lower panel) in the anterior compartment show high levels of full-length Ci (red), consistent with a role for CK1 and Roc1a in Ci processing.

4 Publications

4.1 Journals

Wang, Y. & Price, M.A. A unique protection signal in Cubitus interruptus prevents its complete proteasomal degradation. *Mol Cell Biol* 28 (18), 5555-5568 (2008).

4.2 Book(s) and other one-time publications

Nothing to report.

4.3 Oral presentations

Price, M. A., Miyagi, C., Hasegawa, S., Lehmann, I. T., Azama, C. DNA: the Blueprint of Life, OIST Open House, OITC, Okinawa Japan, Nov. 9, 2009

Price, M. A., Spratt, S., Hussein, Y. S., Miyagi, C., Hasegawa, S. Green Fluorescent Protein, OIST Open House, OITC, Okinawa Japan, Nov. 9, 2009

Price, M. A., Wang, Y., Isoe, J. The Mechanism of Ci Processing, Gradients and Signalling Workshop, OIST Seaside House, Okinawa, Japan, Nov. 18, 2009

Price, M. A., Vickers, C., Hasegawa, S., Arai, M., Azama, C. DNA: the Blueprint of Life, Kubura Junior High School, Yonaguni, Okinawa-ken, Japan, Feb. 5, 2009

Price, M. A., Vickers, C., Hasegawa, S., Arai, M., Azama, C. DNA: the Blueprint of Life, Yonaguni Junior High School, Yonaguni, Okinawa-ken, Japan, Feb. 6, 2009

4.4 Posters

Price, M. A., Wang, Y., Hasegawa, S. Mechanism of the partial proteolysis of Cubitus interruptus, "The Many Faces of Ubiquitin" Keystone Conference, Copper Mountain, CO, USA, January 11-16, 2009

Price, M. A., Wang, Y., Hasegawa, S. Mechanism of the partial proteolysis of Cubitus interruptus, 50th Annual U.S. Drosophila Research Conference, Chicago, IL, USA, March 4-8, 2009

5 Intellectual Property Rights and Other Specific Achievements

Nothing to report.

6 Meetings and Events

6.1 Gradients and Signalling: from chemotaxis to development

Date: November 17-21, 2009
Venue: OIST Seaside House
Co-organizers: Dr. Ichi Maruyama, Dr. Ichiro Masai, Dr. Fadel Samatey
Speakers: Dr. Konrad Basler (University of Zurich, Switzerland)
Dr. Philip Beachy (Stanford University, Palo Alto, USA)
Dr. Ariane Briegel (Cal Tech, Pasadena, USA)
Dr. James Briscoe (NIMR, London, UK)
Dr. Brian Crane (Cornell University, NY, USA)
Dr. Joe Culotti (University of Toronto, Canada)
Dr. Matthew Freeman (MRC-LMB, Cambridge, UK)
Dr. Hiroshi Hamada (Osaka University, Japan)
Dr. Xi He (Harvard Medical School, Boston, USA)
Dr. Caroline Hill (CR-UK, London, UK)
Dr. Ikuro Kawagishi (Hosei University, Tokyo, Japan)
Dr. Maria Leptin (University of Koln, Germany)
Dr. Sandy Parkinson (University of Utah, USA)
Dr. Mu-ming Poo (UC Berkeley, USA)
Dr. Mary Ann Price (OIST, Japan)
Dr. Sudipto Roy (ICMB, Singapore)
Dr. Matthew Scott (Stanford University, Palo Alto, USA)
Dr. Victor Sourjik (Heidelberg University, Germany)
Dr. Tetsuya Tabata (University of Tokyo, Japan)
Dr. Shin-ichiro Takahashi (University of Tokyo, Japan)
Dr. Jean-Paul Vincent (NIMR, London, UK)
Dr. Wenqing Wu (University of Washington, Seattle, USA)

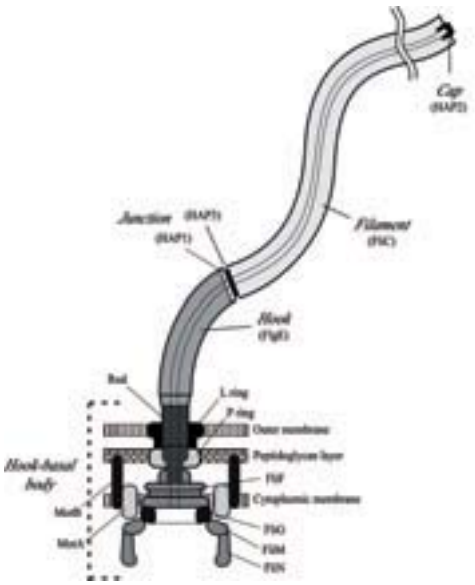
Trans-membrane Trafficking Unit

Principal Investigator:
Fadel A. Samatey

Research Theme:
Functional study of bacterial secretion systems

Abstract

Motility is a very important function in the living world. For this purpose, organisms such as bacteria have developed the most incredible molecular machine: the flagellar system. Bacteria such as *Escherichia coli* and *Salmonella typhimurium* swim by rotating long helical filaments called the flagellum. The flagellum is a complex structure made by the association of many different proteins. It can be divided into three parts: 1) the filament: a long tubular structure that works as a helical propeller, 2) the hook: a short, highly flexible tubular segment that works as a universal joint, 3) the basal body: a rotary motor embedded in the cell membrane.
During the construction of the flagellum, all the flagellar axial proteins are exported from the cytoplasm to the flagellum distal end through a 2 -3 nm channel located at its centre. This export mechanism is regulated by a specialized protein export system located on the cytoplasmic side of the basal body. It is called the type III export apparatus and is found throughout the bacterial kingdom. In the case of *Salmonella*, this export apparatus is made by six membrane proteins: FlhA, FlhB, FliO, FliP, FliQ, FliR, and three cytoplasmic proteins: FliI, FliH and FliJ. The export apparatus of the bacterial flagellum is homologus to the type III secretion system (T3SS) found in Gram-negative pathogenic bacteria. The T3SS role is to secrete virulence factors to host cells, leading to diverse diseases. To understand both the bacterial flagellum and its export apparatus, we have been doing structural studies on some flagellar proteins and genetic studies on the export apparatus. Some of the results are being presented here.



Schematic view of the flagellum

1 Staff

Trans-membrane Trafficking Unit

Researchers: Clive S. Barker
Vladimir Meshcheryakov
Hideyuki Matsunami
Emma-Karin Millers

Technical Staff: Irina Meshcheryakova
Yasuji Kido

Research Administrator / Secretary: Saeko Hedo

2 Partner Organizations

Graduate School of Frontier Biosciences, Osaka University, Japan
Type of partnership: Joint research
Name of principal researcher: Prof. Keiichi Namba
Name of researchers: Keiichi Namba, Katsumi Imada
Research theme: Expression and purification of membrane protein complex

Institut Laue-Langevin, Grenoble, France
Type of partnership: Collaboration
Name of principal researcher: Dr. Giuseppe Zaccaï
Research theme: Molecular dynamic of the bacterial flagellum by neutron scattering

CNRS, Institute of Structural Biology and Microbiology (IBSM), Marseille, France
Type of partnership: Collaboration
Name of principal researcher: Dr. Jean-Romé Voulhoux
Name of researchers: Jean-Romé Voulhoux, Gérard Michel
Research theme: Structural Study of the bacterial Type II secretion system

Robert Wood Johnson Medical School, New Jersey, USA
Type of partnership: Collaboration
Name of principal researcher: Dr. Alla Kostyukova
Name of researcher: Alla Kostyukova
Research theme: Structural investigation of disordered proteins

Institut de Biologie Physico-Chimique, CNRS, Paris, France
Type of partnership: Collaboration
Name of principal researcher: Dr. Bruno Miroux
Name of researchers: Bruno Miroux, Jean-Luc Popot, Francesca Zito
Research theme: *In vitro*, *in vivo* high-yield production of membrane proteins

3 Activities and Findings

3.1 Crystallization of the periplasmic chaperone protein FlgA essential for flagellar P-ring assembly in *Salmonella*

The bacterial flagellum, a cell surface organelle embedded in both membranes, consists of the filament, the hook and the basal body. The basal body is divided into three sub-structures, the LP-ring as a molecular bushing, the MS-ring as a base plate and the rod as a drive shaft connecting the hook and the MS-ring. FlgH and FlgI are the structural subunit components of the L- and P-rings, respectively. FlgA is involved in flagellar P-ring assembly. In the absence of FlgA, basal bodies lacking the LP-ring are produced. The L-ring formation followed by P-ring assembly is a checkpoint for outer membrane penetration for the growing flagellum. FlgH, FlgI, and FlgA, are the only flagellar proteins that have signal sequences in their N-termini for secretion into the periplasm by the general secretion pathway. To reveal the molecular basis for flagellar P-ring assembly, structural models of FlgA would provide valuable clues for better understanding.

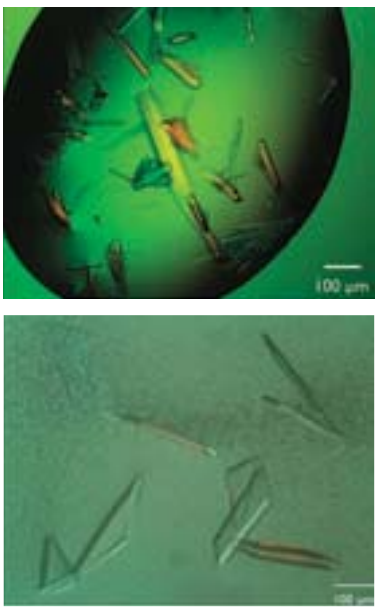


Figure 1: Crystals of FlgA-His (*top*) and SeMet-substituted Crystals of FlgA-His (*bottom*).

We have cloned the full-length *flgA* gene from *Salmonella enterica* serovar Typhimurium and overproduced it as a C-terminally His-tagged fusion protein in *E. coli* BL21(DE3). The protein was successfully released into the periplasmic fraction of the cells and purified by nickel chelating chromatography and followed by

size-exclusion chromatography. Initial crystallization screening was performed by the sitting-drop vapor diffusion method using commercially available screening kits.

Clusters of thin needle-shaped crystals were grown at 16 ℃. Diffraction quality crystals were grown by the hanging-drop vapor diffusion method after optimization of the condition by varying the pH of the crystallization buffer and the concentration of the precipitant. SeMet-substituted crystals were also prepared for phasing. All diffraction data were collected at BL41XU SPring-8. Under cryogenic temperature, native crystals of FlgA-His diffracted beyond 2.0 Å resolution and SeMet-substituted FlgA-His crystals also diffracted to 2.5 Å. All data are processing for structural determination of FlgA.

3.2 Crystallization of a C-terminal fragment of FlgJ, a putative flagellar rod cap protein FlgJ from *Salmonella*.

For the completion of the flagellar rod assembly, it must penetrate the rigid structure of bacterial peptidoglycan. FlgJ is a putative flagellar rod capping protein with peptidoglycan hydrolysing activity, deduced from its sequence similarity. FlgJ has an N-terminal flagellar domain indispensable for rod formation and a C-terminal enzymatic domain that shares a sequence similarity to muramidase, such as autolysin, muramidase2 and AcmA. An understanding of the molecular mechanisms that underlie rod formation requires structural information of FlgJ.

Since *Salmonella* FlgJ aggregated in crystallization conditions and had been recalcitrant to crystallization in its full-length form, we identified suitable fragments for crystallization. A fragment consisting residues from 121-316 of FlgJ (hereafter designated as $FlgJ^{121to316}$), encompassing the C-terminal muramidase domain, has been over-expressed and purified under denatured condition by high concentration of urea because of its protease sensitivity. Initial crystals were grown by sitting-drop vapor diffusion methods using crystallization screening kits. Diffraction quality crystals of $FlgJ^{121to316}$ were obtained by PEG 3350 as a precipitant at 4℃ and belonged to the orthorhombic space group $P2_12_12_1$ with unit-cell parameters, a = 38.8, b = 43.9, c = 108.5 Å. SeMet-substituted $FlgJ^{121to316}$ crystals were isomorphous with the native crystal and MAD data collection from single crystal was also successful. These diffraction data were collected at Spring-8 BL38B1 and BL41XU and structure determination of $FlgJ^{121to316}$ by using MAD data is in progress.

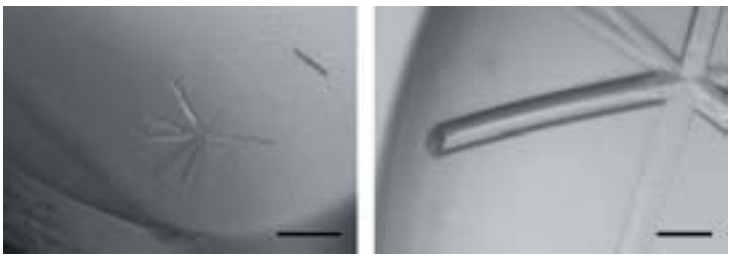


Figure 2: Crystals of $FlgJ^{120-316}$-His. The left panel shows thin needle crystals obtained from initial screening. The left panel shows crystals of $FlgJ^{120-316}$. Rod shaped crystals of $FlgJ^{120-316}$ obtained after optimization. Scale bar, 0.1 mm.

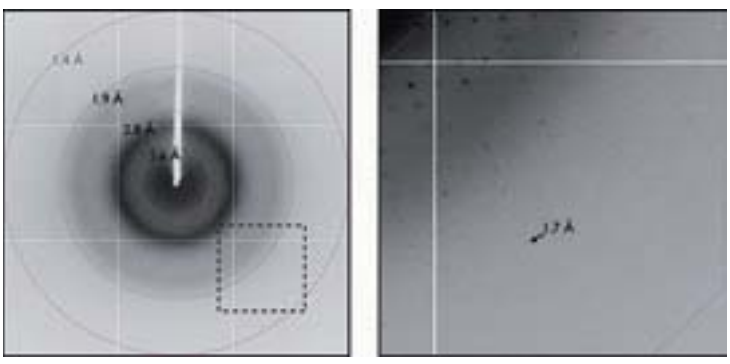


Figure 3: On the left panel, the diffraction pattern of $FlgJ^{120-316}$ crystal collected on ADSC Quantum 315 at BL41XU SPring-8. The crystal exposed to X-ray for 1 sec at an oscillation angle of 1°. Resolution circles were colored by *magenta*. The area enclosed by the *dashed line* was enlarged for clarity. A diffraction spot corresponding to 1.7Å resolution was indicated by *arrowhead* (right panel).

3.3 Functional study of the type III secretion system protein FlhB:

As stated earlier, the T3SSs of bacterial flagella are found throughout the bacterial kingdom. FlhB protein orthologues are present in all flagellar T3SSs, and also its paralogues are present in the T3SSs of all virulence-associated bacterial needles. The sequence composition is very highly conserved for all homologues. Such a library of orthologues of the FlhB protein provides a powerful tool to investigate the effect of multiple point mutations on the *S. typhimurium* FlhB protein, and can be applied in combination with standard analyses such as site-directed mutagenesis. Presumably, the FlhB proteins in each species have a similar function and so the changes are likely to be confined to spatial organization of the protein, and to protein to protein binding interactions rather than catalytic mechanism.

As a starting point, we wished to investigate the complementation properties of a FlhB orthologue with relatively low sequence identity to that of *S. typhimurium*, rather than start with a protein, which may partially complement from the beginning. From an analysis of the evolutionary relationships of the bacterial kingdom, the hyperthermophilic bacterium *Aquifex aeolicus* is one of the most distantly related to *S. typhimurium*. Many proteins this organism produces are studied due to their heat stable nature. The FlhB protein of *A. aeolicus* shares a 32% sequence identity over its length with *S. typhimurium* FlhB, which is amongst the lowest of the orthologues of *S. typhimurium* FlhB. FlhB consists of a predominately transmembrane N-terminal domain and a cytoplamic C-terminal domain. The C-terminal domain is more highly conserved for FlhB homologues (C. Barker and F. A. Samatey, unpublished observations). Chimeras of *S. typhimurium* FlhB and *A. aeolicus* FlhB N-terminal and C-terminal domains, are also interesting to examine since an *S. typhimurium* FlhB (1-218)-*A. aeolicus* FlhB (213-350) chimera has a 78% identity, and an *A. aeolicus* FlhB (1-212)-*S. typhimurium* FlhB chimera (219-383) has a 57% identity.

Using a *S. typhimurium flhB* null mutant as a genetic background we have complemented the strain with full-length FlhB protein form *A. aeolicus*, and chimeras of *S. typhimurium* FlhB and *A. aeolicus* FlhB N-terminal and C-terminal domains. We have investigated the ability of the complemented mutant to swim through semi-solid agar medium (see photos below). As can be seen full-length FlhB from *A. aeolicus* shows a leaky phenotype in comparison to a negative control after extended incubation. However, both N-terminal domain from *A. aeolicus* FlhB/C-terminal domain from *S. typhimurium* FlhB, and N-terminal domain from *S. typhimurium* FlhB/C-terminal domain from *A. aeolicus* FlhB chimeras are even more leaky and are able to form motile mutants containing suppressor mutations after extended incubation in semi-solid medium.

Colony purification and subsequent sequencing has shown the suppressors are not occurring within the chimeric *flhB* gene (I. Meshcheryakova and F. A. Samatey, unpublished observations). Genetic mapping should be able to identify the location of the suppressor mutations on the chromosome, which will give clues to the interaction sites of FlhB with other proteins, and will allow us to follow up with protein interaction binding assays.

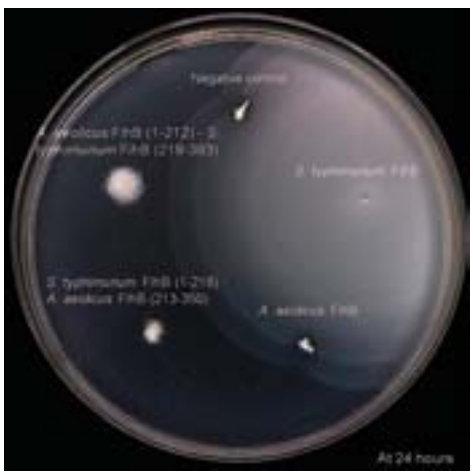


Figure 4: A *S. typhimurium flhB* null mutant complemented by *S. typhimurium* FlhB, *A. aeolicus* FlhB, and *S. typhimurium*/*A. aeolicus* FlhB chimeras

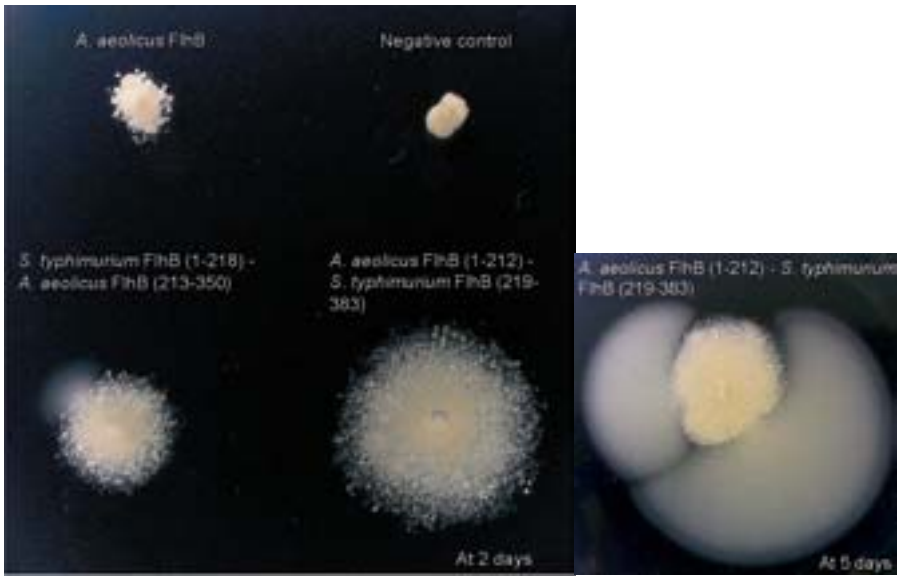


Figure 5: Extended incubation in swarm agar identifies suppressor mutants of *S. typhimurium*/*A. aeolicus* FlhB chimeras

4 Publications

4.1 Journals

Kikuchi, Y., Matsunami, H., Yamane, M., Imada, K., & Namba, K. Crystallization and preliminary X-ray analysis of a C-terminal fragment of FlgJ, a putative flagellar rod cap protein from Salmonella. *Acta Crystallogr Sect F Struct Biol Cryst Commun* 65 (Pt 1), 17-20 (2009).

4.2 Book(s) and other one-time publications

Nothing to report.

4.3 Oral presentations

Samatey, F.A., Structure and mechanism of the flagellar universal joint, "Neutrons and X-rays meet biology", Helmholtz Zentrum, Berlin, Germany, February 25-27, 2009

Samatey, F.A., Structural study of membrane proteins, Institut de Biologie Physico-Chimique, Paris, France, March 3, 2009

4.4 Posters

Nothing to report.

5 Intellectual Property Rights and Other Specific Achievements

Nothing to report.

6 Meetings and Events

6.1 OIST Workshop: Protein 3D Structure Visualization and Structural Bioinformatics

Date: May 13, 2008
Venue: IRP conference room, OIST, Uruma
Speaker: Prof. Eric Martz, Dept. of Microbiology, University of Massachusetts, Amherst, USA

6.2 OIST Seminar

Date: October 28, 2008
Venue: IRP conference room, OIST, Uruma
Speaker: Dr. Md. Imtaiyaz Hassan, Center for Interdiciplinary Research Basic Science

6.3 OIST Seminar

Date: November 14, 2008
Venue: IRP conference room, OIST, Uruma
Speaker: Dr. Emma-Karin Millers, School of Molecular and Microbial Sciences, University of Queensland, St Lucia, Australia

6.4 OIST Workshop: Gradients and Signalling: from chemotaxis to development

Date: 17-21 November, 2008
Venue: Seaside House, OIST, Onna, Okinawa, Japan
Co-organizers: Dr. Maruyama, Dr. Masai, Dr. Price

Marine Genomics Unit

Principal Investigator:
Nori Satoh

Research Theme:
Evolutionary, Developmental, and Environmental Genomics of Marine Invertebrates

Abstract

Recent decoding of genome sequences of various animals around the turn of the 21st century has promoted genome sciences and systems biology in the realm of biological sciences of the new era. Especially, gene regulatory networks that consist of subcircuits of transcription factors and intercellular signaling molecules are key to an understanding of the complex mechanisms of animal development and evolution. The objective of our research is to elucidate genome-widely molecular mechanisms underlying the evolution, development, and environment responses, mainly using marine invertebrate chordates. The major achievements of my Unit in this fiscal year 2008 are as follows.

(a) **Evolutionary genomics:** We are interested in the origin and evolution of chordates. The phylum Chordata consists of urochordates (ascidians), cephalochordates (amphioxus), and vertebrates or ourselves. To investigate the evolution of chordates, we decoded in 2002 the genome of the urochordate *Ciona intestinalis.* This year, 2008, we have decoded the genome of the cephalochordate *Branchiostoma floridae.* Comparison of the genomes with those of echinoderms and vertebrates clearly showed that cephalochordates are basal among chordates, suggesting a free-living ancestor of chordates. In addition, it became clear that the amphioxus genome has retained a high level of synteny with vertebrate genomes, allowing us reconstruction of the gene compliment of the last common chordate ancestor.

(b) **Developmental genomics:** The objective of this part of research is to elucidate the gene regulatory networks in development, using *C. intestinalis. Ciona intestinalis* was the seventh assembled animal genome, that is composed of just 160 Mbp and contains ~16,000 protein-coding genes. We focus on 390 core transcription factor genes and 119 major signaling molecule genes in this animal genome. This year we mapped almost all of the genes on the 14 pairs of *Ciona* chromosomes to examine chromosomal localization of upstream and downstream regulatory genes that are responsible for the construction of chordate body plan. This study demonstrated that the components of coherent developmental gene networks are evenly distributed over the 14 chromosomes, providing the first comprehensive evidence that the physical clustering of regulatory genes is not relevant for the genome-wide control of gene expression during development.

(c) **Environmental genomics:** We are performing comprehensive analyses of gene regulatory networks that underlie responses to various environmental cues. The research promises to greatly advance our understanding of how marine organisms survive environmental changes. Two major researches are now undergoing, first decoding of the genomes of corals and their symbionts, and second, analysis of *Ciona* genes which respond to environmental stresses.

1 Staff

Researchers: Eiichi Shoguchi, Takeshi Kawashima, Mayuko Hamada
Technical Staff: Chuya Shinzato, Manabu Fujie, Kanako Hisata
Research Administrator / Secretary: Ken Maeda

2 Partner Organizations

University of California, Berkeley
Type of partnership: Collaboration
Name of principal researcher: Daniel S. Rokhsar
Name of researcher: Nickolas H. Putnam
Research theme: Decoding of the amphioxus genome

National Institute for Basic Biology, Japan
Type of partnership: Collaboration
Name of principal researcher: Hiroki Takahashi
Research theme: Chip-sequence analysis of *Ci-Bra* target genes

Shimoda Marine Research Center, University of Tsukuba
Type of partnership: Collaboration
Name of principal researcher: Yasunori Sasakura
Research theme: Analysis of *Ciona* genes with transgenic lines

Graduate School of Life and Environmental Sciences, Univ. of Tsukuba
Type of partnership: Collaboration
Name of principal researcher: Hiroshi Wada
Research theme: Analysis of the evolution of chordate genomes

3 Activities and Findings

3.1 Evolutionary Genomics:

The phylum Chordata consists of the subphyla Cephalochordata (amphioxus), Urochordata (ascidians) and Vertebrata. These three groups are characterized by possession of a notochord, a dorsal hollow neural tube or central nervous system, branchial slits, an endostyle, and a postnatal tail, the first two being hallmarks of the chordate body plan. The chordates are also members of the animal group deuterostomes, together with echinoderms (starfish and sea urchins) and hemichordates (acorn worms). The chordates are thought to have evolved from a common ancestor shared with the two non-chordate deuterostome phyla. The boundary between chordates and non-chordate invertebrates looks deep into evolutionary history, suggesting that both hemichordates and echinoderms are important out-groups to consider the evolution of chordates.

Genomes of several vertebrates including human being have been sequenced since 2001. In 2002, in collaboration with Joint Genome Institute (JGI), DOE, USA and others, we decoded the draft genome of the urochordates *Ciona intestinalis.* Although analysis of the *Ciona* genome suggested that ascidians contain the basic ancestral complement of genes involved in cell signaling and development and that the ascidian genome has also acquired a number of lineage-specific innovation, it did not necessarily provide significant information on the origin and evolution of chordates. Therefore, in collaboration with JGI, we have conducted decoding of the genome of the cephalochordate *Branchiostoma floridae* since 2004, and this year we finally obtained the result (Figure 1). Namely, comparison of the primitive chordate genomes with those of invertebrates and vertebrates suggests that cephalochordates are basal among the chordates, with urochordates sister to vertebrates. Because amphioxus are free-living chordates, this result suggests a free-living ancestor of chordates. In addition, it became clear that the amphioxus genome has retained a high level of synteny with vertebrate genomes, allowing us reconstruction of the gene compliment of the last common chordate ancestor (Figure 2).

Figure 1: Cover of "Nature, Vol. 453, No. 7198" to report the amphioxus genome.

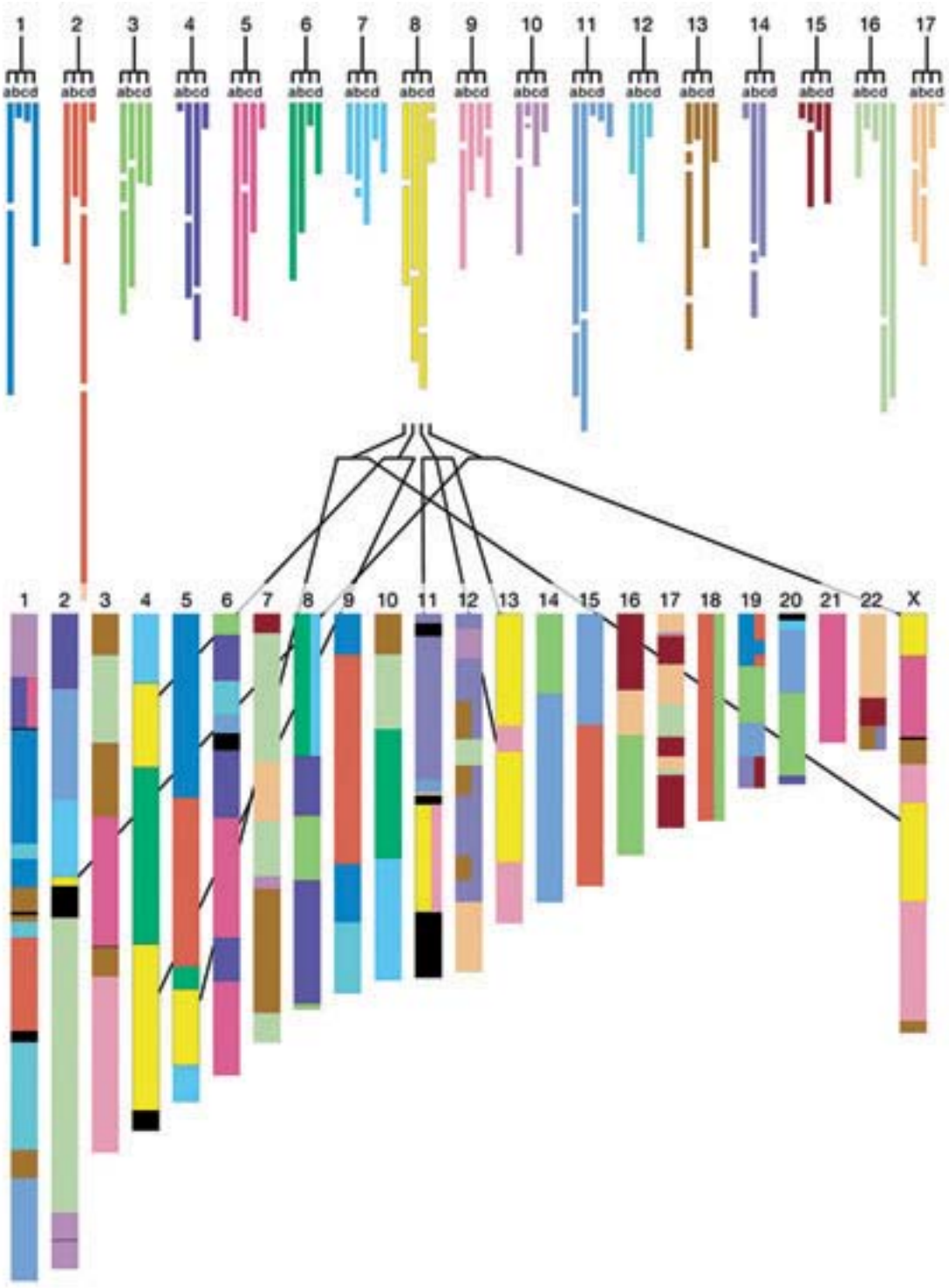


Figure 2: Quadruple conserved synteny. Partitioning of the human chromosomes into segments with defined patterns of conserved synteny to amphioxus (*B. floridae*) scaffolds. Numbers 1–17 at the top represent the 17 reconstructed ancestral chordate linkage groups, and letters a–d represent the four products resulting from two rounds of genome duplication. Coloured bars are segments of the human genome, shown grouped by ancestral linkage group (above), and in context of the human chromosomes (below).

The origin and evolution of the chordates has been the subject of extensive investigation and speculation for more than one and half centuries. As described above, comparison of the chordate and non-chordate genomes demonstrated that cephalochordates are basal among the chordates, suggesting a free-living ancestor of chordates. In order to explain how chordates evolved from common ancestors shared with non-chordate deuterostomes, N. Satoh has proposed an aboral-dorsalization hypothesis (Figure 3). This hypothesis is based on the notions that (1) cephalochordates are evolutionarily closest to the chordate ancestor, (2) the occurrence of a tadpole-type larva with a neural tube and notochord was essential for creation of the chordate body plan, (3) these two structures are completely novel without any relation to any structures of non-chordate deuterostomes, (4) the morphogenesis to form the two structures occurs during embryogenesis, and (5) due to the limited space in the oral side of the ancestral embryo, these events occurred in the aboral side of the embryo. The hypothesis should be discussed further with molecular developmental biology data in the future.

In addition, N. Satoh has proposed an advanced filter-feeder hypothesis to explain how the huge variety of lifestyles of extant urochordates evolved from a cephalochordate-like ancestor. Although the taxonomic position of larvaceans is enigmatic, it is argued that among urochordates, free-living larvaceans are basal, while sessile ascidians are derived. All the urochordate traits might have been evolved in response to strong evolutionary advantages as specialists of suspension filter feeding. Nevertheless, the molecular mechanisms involved in the formation of the notochord and several other organs in ascidian embryos are more comparable to those of vertebrates than cephalochordates. Thus, urochordates are close relatives to vertebrates. Together with the aboral-dorsalization hypothesis, this hypothesis provides an opportunity to discuss further the origin and evolution of chordates.

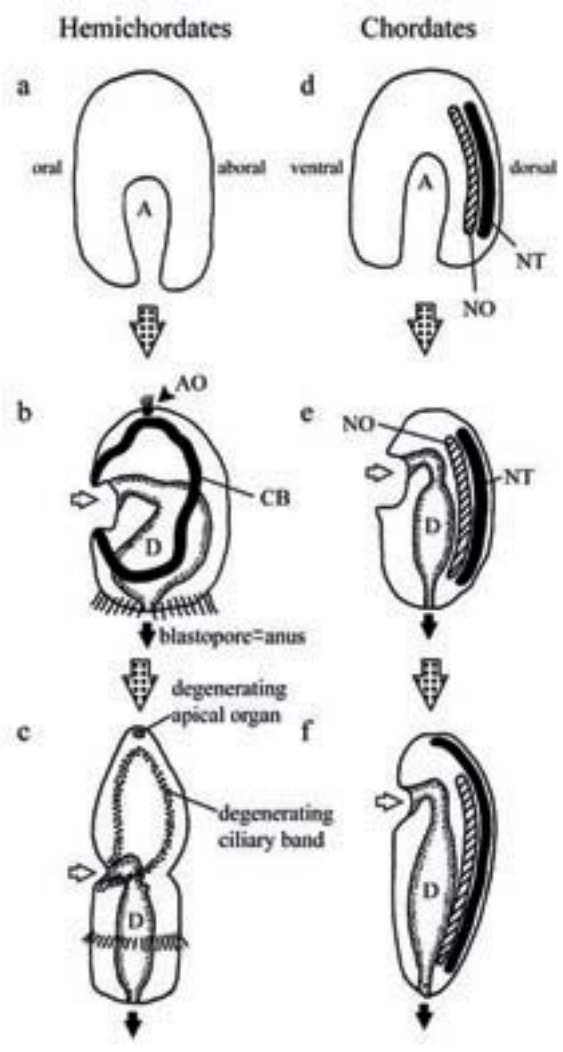


Figure 3: A schematic drawing showing the essence of the aboral dorsalization hypothesis to explain the creation of tadpole-type larvae with a dorsal neural tube and notochord, during the evolution of chordates. (a–c) Embryogenesis of hemichordates and (d–f) that of chordates. It should be emphasized here that the neural tube and notochord that are formed on the dorsal side of the embryo are completely novel structures without any relationship to the apical organ and stomochord of hemichordate enteropneust embryos and juveniles, respectively. A, archenteron; AO, apical sensory organ; CB, ciliary band; D, digestive track; NT, neural tube; NO, notochord. White arrows indicate mouth and black arrows anus.

3.2 Developmental Genomics:

The final goal of this research is to describe in detail the gene regulatory networks that are responsible for the formation of the chordate body plan. We wish to discover a general principle or dogma that governs the complex genetic and/or genomic process of embryological development. In this respect, *Ciona intestinalis* provides an attractive experimental system in the field of developmental genomics. Fertilized eggs of *C. intestinalis* develop rather quickly into so-called tadpole-type larvae. The ascidian tadpole is composed of only ~2600 cells, which constitute a small set of larval organs including the epidermis, central nervous system, notochord and tail muscle along with the rudiments of the adult gut, mesodermal organs and gonads. This configuration, with the dorsal nervous system overlying a notochord, represents the basic chordate body plan.

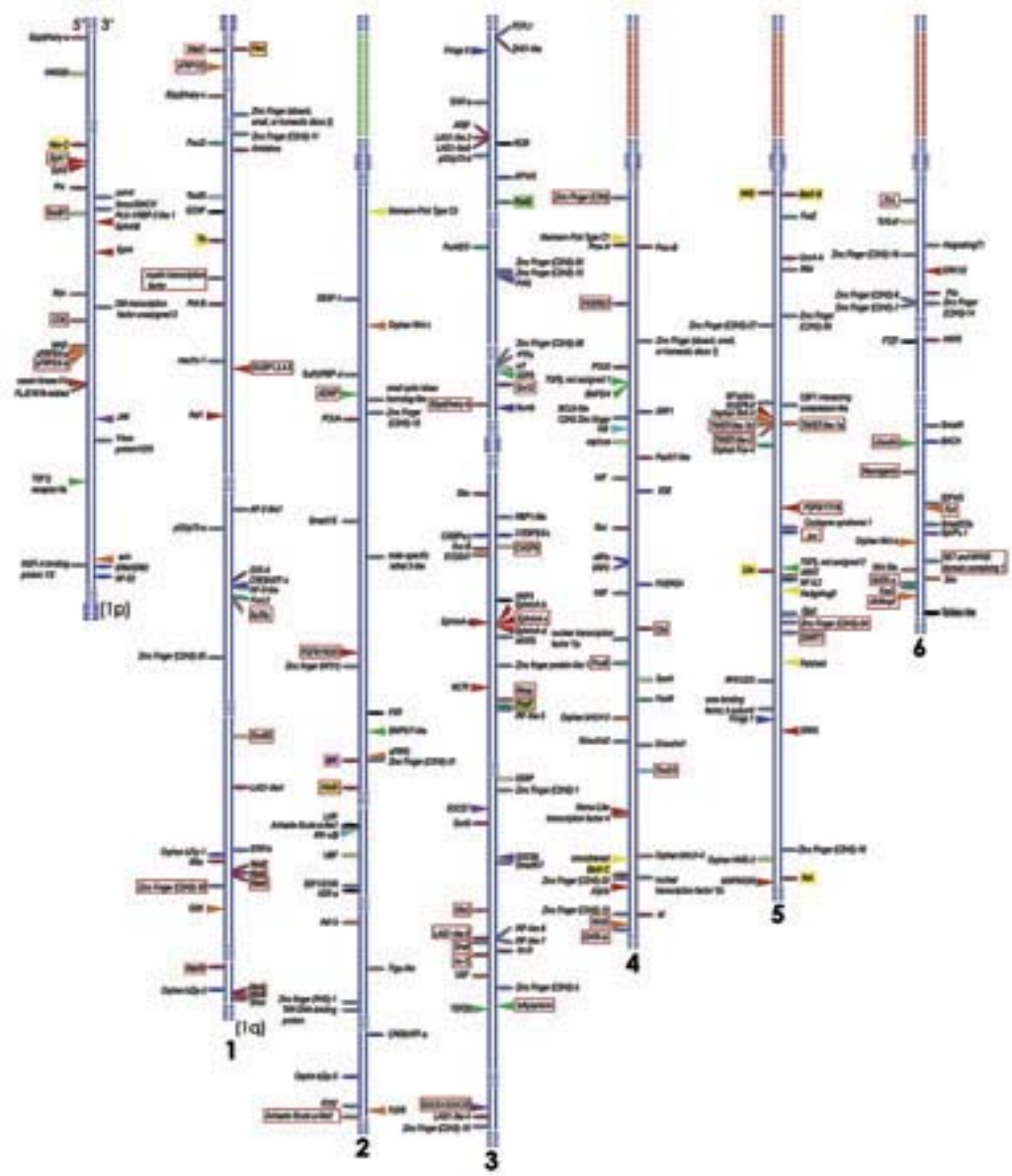


Figure 4: Chromosomal map of 373 core transcription factor genes and 111 major cell signaling molecule genes in *Ciona intestinalis*. Families of transcription factors are shown by discs with different colors; cell signaling molecules are shown by arrowheads with different colors (bottom-right corner). Centromeric regions are shown by dark blue dashed-lines. Red dashed-lines indicate three rDNA cluster regions, green-dashed lines a histone cluster region. Blue dashed-lines indicate unmapped regions. The left and right vertical lines of each chromosome indicate the 5' to 3' and 3' to 5' alignment, respectively. The telomeric regions on the short arms of chromosomes 12, 13 and 14 are ordered arbitrarily. Clustered genes in other chordate genomes are shown using color code. The enclosed genes in red lines are those analyzed as elements of the regulatory network for chordate body plan construction (Figure. 5).

Ciona intestinalis was the seventh assembled animal genome, that is composed of just 160 Mbp and contains ~16,000 protein-coding genes. The *Ciona* genome contains 669 transcription factor (TF) genes and more than several hundred signaling molecule (ST) genes. Our previous studies demonstrated that ~360 core TF genes and ~120 major ST genes are involved in the development of *Ciona* larvae. We focus on 390 core transcription factor genes and 119 major signaling molecule genes in further studies. This year we mapped approximately 82% of the genome sequence information on all arms of the 14 pairs of the *Ciona* chromosomes (Figure 4), including 373 TF genes and 111 ST genes.

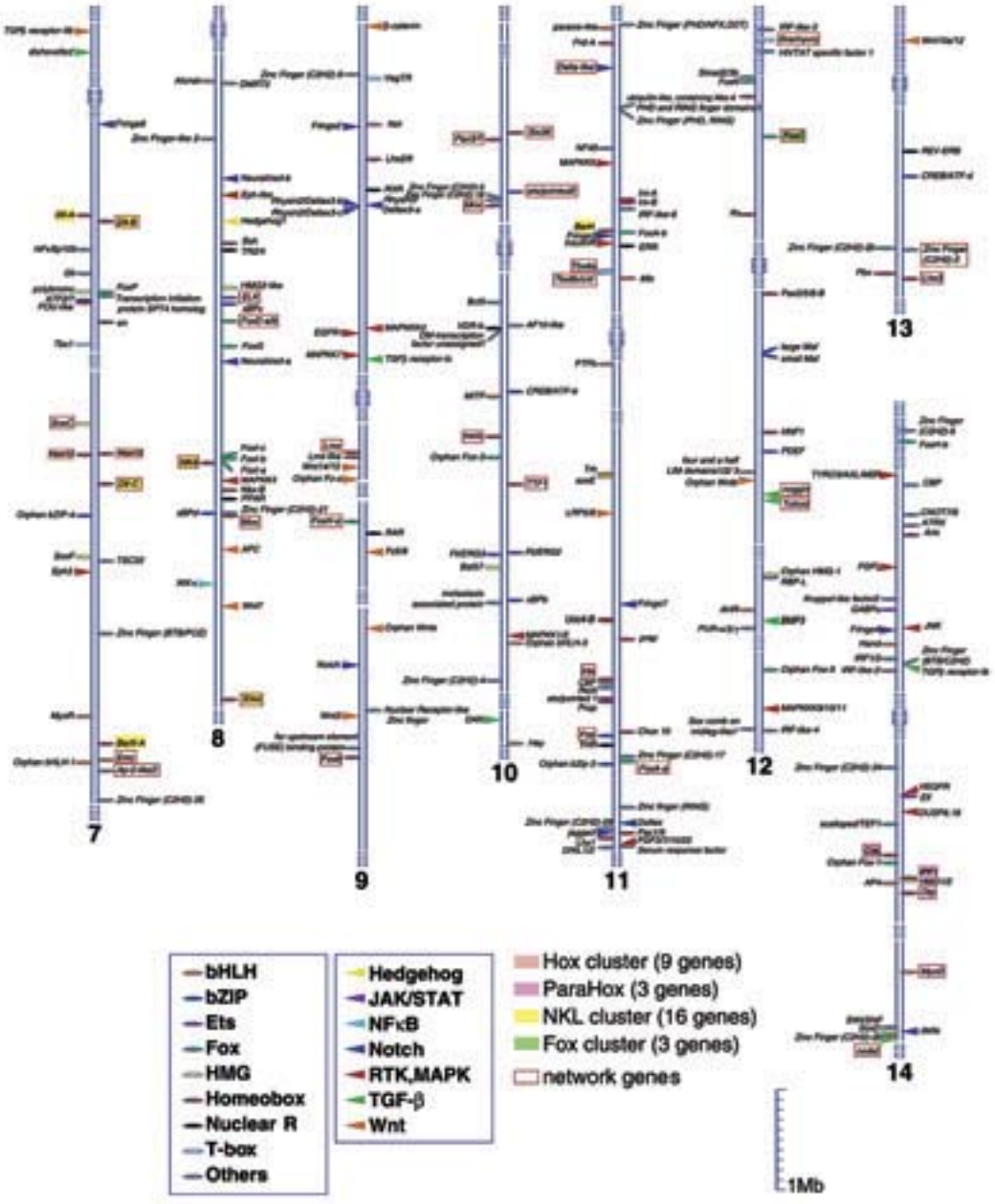


Figure 4: (continued).

Previous gene disruption assays led to the formulation of a basic chordate embryonic blueprint, based on over 3000 genetic interactions among 79 zygotic regulatory genes. We mapped all 79 regulatory genes on the 14 pairs of *Ciona* chromosomes to examine chromosomal localization of upstream and downstream regulatory genes that are responsible for the construction of chordate body plan (Figure 5). This study demonstrated that the components of coherent developmental gene networks are evenly distributed over the 14 chromosomes, providing the first comprehensive evidence that the physical clustering of regulatory genes is not relevant for the genome-wide control of gene expression during development.

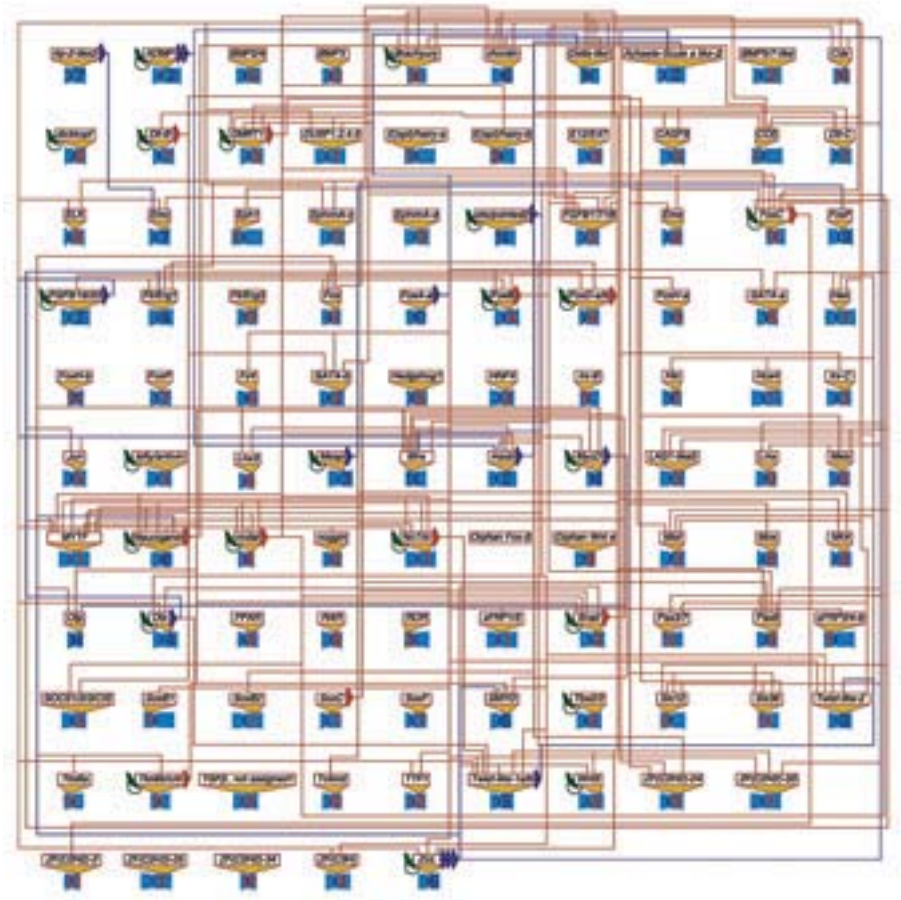


Figure 5: The chromosomal localization of *Ciona* genes involved in the regulatory network responsible for the specification of embryonic cells. The genes are ordered alphabetically from the top-left corner to the bottom-right corner. The chromosomal localization of genes is shown in light blue boxes, with short (left column) and long (right) arms. Only orphan Fox-2 was not characterized. Lines show gene networks, dark blue lines and arrows for same chromosome interactions, orange lines for inter-chromosomal interactions, and green arrows for auto-regulatory interactions. Same chromosome interactions are highlighted.

3.3 Environmental Genomics:

Every marine organism has to adapt to environment changes, which are not always comfortable to them. For example, drastic changes in seawater temperature or salinity and pollution with unfavorable factors for living organisms might be stressful. However, in order to survive, they have to overcome such changes by altering the gene expression profile or by molecular modification. Such environmental changes have been the subject of physiological, molecular and ecological studies, but have not often been discussed in the context of gene regulatory networks. Two lines of research are now undergoing.

Firstly, we are now decoding coral genomes. Coral bleaching caused by global warming is one of the major threats to coral reefs. Although recently research has focused on the relationship between corals and symbionts at molecular level, it looks insufficient to resolve this problem. Decoding genomes of both coral and symbionts is crucial to extend further studies to exploring the molecular mechanisms underlying coral bleaching. We selected the coral *Acropora digitifera* (Figure 6) as a

target of genome sequencing because this species is most common in Okinawa islands. It was determined that the genome of A. *digitifera* is approximately 420 Mbp in size. Using new-generation sequence machine, 454 Life Sciences (Roche) GS FLX, we have already obtained 6X coverage of the *Acropora* genome. We are now assembling and annotating the genome sequences.
Secondly, using the *Ciona* system, we are examining the changes in gene expression profiles by restricting the study to two environmental factors, temperature and salinity. For each factor, both embryos and adults are exposed to changes in the environmental cue, and two phases of their response, the initial response phase and steady recovery phases, are examined with a 44-K oligonucleotide-based microarray. Microarray analyses will determine changes in the gene expression profiles at each of the two stages. A similar analysis would be carried out with the other factor, temperature, after which we would compare the expression profiles obtained from all experiments to determine whether or not a similar set of genes is involved in the initial response phase and steady recovery phases. In other words, we wish to find a general principle or dogma that governs the complex genetic and/or genomic process in response to environmental changes.

Figure 6: A colony of *Acropora digitifera* used for genome sequencing.

4 Publications

4.1 Journals (including publications done by N. Satoh, E. Shoguchi and M. Hamada at Kyoto Univ., T. Kawashima at UC Berkeley, and C. Shinzato at James Cook University)

Putnam, N.H., Butts, T., Ferrier, D.E., Furlong, R.F., Hellsten, U., Kawashima, T., Robinson-Rechavi, M., Shoguchi, E., Terry, A., Yu, J.K., Benito-Gutierrez, E.L., Dubchak, I., Garcia-Fernandez, J., Gibson-Brown, J.J., Grigoriev, I.V., Horton, A.C., de Jong, P.J., Jurka, J., Kapitonov, V.V., Kohara, Y., Kuroki, Y., Lindquist, E., Lucas, S., Osoegawa, K., Pennacchio, L.A., Salamov, A.A., Satou, Y., Sauka-Spengler, T., Schmutz, J., Shin, I.T., Toyoda, A., Bronner-Fraser, M., Fujiyama, A., Holland, L.Z., Holland, P.W., Satoh, N., & Rokhsar, D.S. The amphioxus genome and the evolution of the chordate karyotype. *Nature* 453 (7198), 1064-1071 (2008).

Holland, L.Z., Albalat, R., Azumi, K., Benito-Gutierrez, E., Blow, M.J., Bronner-Fraser, M., Brunet, F., Butts, T., Candiani, S., Dishaw, L.J., Ferrier, D.E., Garcia-Fernandez, J., Gibson-Brown, J.J., Gissi, C., Godzik, A., Hallbook, F., Hirose, D., Hosomichi, K., Ikuta, T., Inoko, H., Kasahara, M., Kasamatsu, J., Kawashima, T., Kimura, A., Kobayashi, M., Kozmik, Z., Kubokawa, K., Laudet, V., Litman, G.W., McHardy, A.C., Meulemans, D., Nonaka, M., Olinski, R.P., Pancer, Z., Pennacchio, L.A., Pestarino, M., Rast, J.P., Rigoutsos, I., Robinson-Rechavi, M., Roch, G., Saiga, H., Sasakura, Y., Satake, M., Satou, Y., Schubert, M., Sherwood, N., Shiina, T., Takatori, N., Tello, J., Vopalensky, P., Wada, S., Xu, A., Ye, Y., Yoshida, K., Yoshizaki, F., Yu, J.K., Zhang, Q., Zmasek, C.M., de Jong, P.J., Osoegawa, K., Putnam, N.H., Rokhsar, D.S., Satoh, N., & Holland, P.W. The amphioxus genome illuminates vertebrate origins and cephalochordate biology. *Genome Res* 18 (7), 1100-1111 (2008).

Satoh, N. An aboral-dorsalization hypothesis for chordate origin. *Genesis* 46 (11), 614-622 (2008).

Satoh, N., An advanced filter-feeder hypothesis for urochordate evolution. *Zoolog Sci* 26 (2), 97-111 (2009).

Srivastava, M., Begovic, E., Chapman, J., Putnam, N.H., Hellsten, U., Kawashima, T., Kuo, A., Mitros, T., Salamov, A., Carpenter, M.L., Signorovitch, A.Y., Moreno, M.A., Kamm, K., Grimwood, J., Schmutz, J., Shapiro, H., Grigoriev, I.V., Buss, L.W., Schierwater, B., Dellaporta, S.L., & Rokhsar, D.S. The Trichoplax genome and the nature of placozoans. *Nature* 454 (7207), 955-960 (2008).

Harada, Y., Takagaki, Y., Sunagawa, M., Saito, T., Yamada, L., Taniguchi, H., Shoguchi, E., & Sawada, H. Mechanism of self-sterility in a hermaphroditic chordate. *Science* 320 (5875), 548-550 (2008).

Christiaen, L., Davidson, B., Kawashima, T., Powell, W., Nolla, H., Vranizan, K., & Levine, M. The transcription/migration interface in heart precursors of *Ciona intestinalis*. *Science* 320 (5881), 1349-1352 (2008).

Shoguchi, E., Hamaguchi, M., & Satoh, N. Genome-wide network of regulatory genes for construction of a chordate embryo. *Dev Biol* 316 (2), 498-509 (2008).

Jeffery, W.R., Chiba, T., Krajka, F.R., Deyts, C., Satoh, N., & Joly, J.S. Trunk lateral cells are neural crest-like cells in the ascidian *Ciona intestinalis*: insights into the ancestry and evolution of the neural crest. *Dev Biol* 324 (1), 152-160 (2008).

Wada, S., Hamada, M., Kobayashi, K., & Satoh, N. Novel genes involved in canonical Wnt/beta-catenin signaling pathway in early *Ciona intestinalis* embryos. *Dev Growth Differ* 50 (4), 215-227 (2008).

Shinzato, C., Iguchi, A., Hayward, D.C., Technau, U., Ball, E.E., & Miller, D.J. Sox genes in the coral *Acropora millepora*: divergent expression patterns reflect differences in developmental mechanisms within the Anthozoa. *BMC Evol Biol* 8, 311 (2008).

Satou, Y., Mineta, K., Ogasawara, M., Sasakura, Y., Shoguchi, E., Ueno, K., Yamada, L., Matsumoto, J., Wasserscheid, J., Dewar, K., Wiley, G.B., Macmil, S.L., Roe, B.A., Zeller, R.W., Hastings, K.E., Lemaire, P., Lindquist, E., Endo, T., Hotta, K., & Inaba, K. Improved genome assembly and evidence-based global gene model set for the chordate *Ciona intestinalis*: new insight into intron and operon populations. *Genome Biol* 9 (10), R152 (2008).

Yu, J.K., Wang, M.C., Shin, I.T., Kohara, Y., Holland, L.Z., Satoh, N., & Satou, Y. A cDNA resource for the cephalochordate amphioxus *Branchiostoma floridae*. *Dev Genes Evol* 218 (11-12), 723-727 (2008).

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Satake, H., Aoyama, M., Kawada, T., Sekiguchi, T., Sakai, T., Fujie, M., & Satoh, N. Neuropeptides and their receptors of the protochordate, *Ciona intestinalis*: the evolutionary origin of vertebrate neuropeptides. *Acta Biol Hung* 59 Suppl, 237-239 (2008).

Aoyama, M., Kawada, T., Fujie, M., Hotta, K., Sakai, T., Sekiguchi, T., Oka, K., Satoh, N., & Satake, H. A novel biological role of tachykinins as an up-regulator of oocyte growth: identification of an evolutionary origin of tachykininergic functions in the ovary of the ascidian, *Ciona intestinalis*. *Endocrinology* 149 (9), 4346-4356 (2008).

Keduka, E., Kaiho, A., Hamada, M., Watanabe-Takano, H., Takano, K., Ogasawara, M., Satou, Y., Satoh, N., & Endo, T. M-Ras evolved independently of R-Ras and its neural function is conserved between mammalian and ascidian, which lacks classical Ras. *Gene* 429 (1-2), 49-58 (2009).

Nakayama-Ishimura, A., Chambon, J.P., Horie, T., Satoh, N., & Sasakura, Y. Delineating metamorphic

pathways in the ascidian *Ciona intestinalis*. *Dev Biol* 326 (2), 357-367 (2009).

Kawashima, T., Hamada, M., Shinzato, C., Satoh, N., & Shoguchi, E. New outlooks on evolutionary studies brought from ten years of animal genome decipherment. *Kagaku* 78, 1070-1079 (2008). (in Japanese)

Satoh, N., Kawashima, T., & Shoguchi, E. Decoding of amphioxus genome and evolution of chordates. *Tanpakushitsu Kakusan Koso* 54 (1), 20-28 (2009). (in Japanese)

4.2 Book(s) and other one-time publications

Kawashima, T., Shoguchi, E., Satou Y., Satoh, N. Comparative genomics of invertebrates. In "Comparative Genomics: Basic an Applied Research" Ed. by J.R. Brown. CRC Press, pp. 87-104. London (2008)

4.3 Oral presentations

Satoh, N. "Developmental Genomics of *Ciona intestinalis*", Plenary Lecture. The 41st Annual Meeting for the Japanese Society of Developmental Biologists. Tokushima Arts Foundation for Culture, Tokushima, Japan, May 28–30, 2008

Satoh, N. "Development of marine genomics in OIST". Genome Renaissance Okinawa Symposium. Okinawa Harborview Crowne Plaza, Okinawa, Japan, June 13, 2008

Kawashima, T. "Ascidian, Amphioxus, then, ... JGI from below". Genome Informatics Workshop 2008. Kazusa Akademia Hall, Chiba, Japan, July 11, 2008

Izumi, M., Fujita, S., Hotta, K., Kawashima, T., Humphreys, T., Satoh, N., Tagawa, K."Expression analysis of Brachyury-downstream genes in the hemichordates, *Ptychodera flava*". 79th Annual Meeting of the Zoological Society of Japan. Fukuoka University, Fukuoka, Japan, September 5–7, 2008

Ikuta, T., Hamada, M., Satou, Y., Satoh, N., Saiga, H. "Analysis of the function of Hox genes in the development of *Ciona intestinalis*". 79th Annual Meeting of the Zoological Society of Japan. Fukuoka University, Fukuoka, Japan, September 5–7, 2008

Satoh, N. "The origin and evolution of Chordates: New insights from developmental genomics". 79th Annual Meeting of the Zoological Society of Japan. Fukuoka University, Fukuoka, Japan, September 5–7, 2008

Satoh, N. "Development of marine genomics in OIST". Meeting of high school principals in Okinawa. Hokubu-Nohrin High School, Okinawa, Japan, October 7, 2008

Kawashima, T. "The genome science in zoology of recent years and the expectations to the Bioinformatics". The 40th SIG-MBI. Japan Advanced Institute Science and Technology, Ishikawa, Japan, March 27–28, 2009

4.4 Posters

Shoguchi, E., Hamaguchi, M., Satoh, N. "Chromosomal mapping of regulatory genes for construction of a chordate embryo". The 41st Annual Meeting for the Japanese Society of Developmental Biologists. Tokushima Arts Foundation for Culture, Tokushima, Japan, May 28–30, 2008

Ikuta, T., Miyamoto, N., Tagawa, K., Humphreys, T., Saito, Y., Satoh, N., Saiga, H. "Isolation of Hox and ParaHox genes in two indirect-developing hemichordate species". 79th Annual Meeting of the Zoological Society of Japan. Fukuoka University, Fukuoka, Japan, September 5–7, 2008

Itoh, N., Shoguchi, E., Satoh, N. "Cytogenetic characterization of the *Branchiostoma floridae* chromosomes". 79th Annual Meeting of the Zoological Society of Japan. Fukuoka University, Fukuoka, Japan, September 5–7, 2008

Shoguchi, E., Fujie, M., Satoh, N. "Chromosomal Level Regulation of Gene Expression in the *Ciona intestinalis* Embryo". The 16th CDB Meeting, "Cis sequence regulation and its evolution". RIKEN Center for Developmental Biology, Kobe, Japan, September 29–Oct 1, 2008

5 Intellectual Property Rights and Other Specific Achievements

None

6 Meetings and Events

6.1 The 35th University of the Ryukyus COE seminar (OIST-COE joint seminar)

Date: May 20, 2008
Venue: University of the Ryukyus, Okinawa, Japan
Co-organizer: Hideo Yamasaki (University of the Ryukyus)
Co-sponsors: OIST
21st COE Program of the University of the Ryukyus
Speakers: Nori Satoh (OIST)
Michio Hidaka (University of the Ryukyus)
Gaku Tokuda (University of the Ryukyus)

6.2 OIST Winter Course "Evolution of Complex Systems"

Date: December 8–14, 2008
Venue: OIST Seaside House, Okinawa, Japan
Co-organizers: Sydney Brenner (OIST)
Nori Satoh (OIST)
Ichiro Masai (OIST)
Mary Ann Price (OIST)
Co-sponsor: OIST
Lecturers: Michael Levine (University of California, Berkeley)
William McGinnis (University of California, San Diego)
David J. Miller (James Cook University)
Richard Harland (University of California, Berkeley)
Sydney Brenner (OIST)
Nori Satoh (OIST)
Speakers in the workshop: Sven Leininger (The University of Bergen)
Shinichi Sunagawa (University of California Merced)
Clement Lamy (Kyoto University)
Martina Hrouda (Kyoto University)
Masa-aki Yoshida (Osaka University)
Shigehiro Yamada (Kyoto University)
Fuki Gyoja (Kyoto University)
Rie Kusakabe (Kobe University)
Kinya G. Ota (Riken CDB)
Koh Onimaru (Tokyo Institute of Technology)
Sukumar Chandra Noskor (Shimane University)
Colin Crist (Institut Pasteur)
Eiji Matsunaga (Riken)
Tsuyoshi Yoda (Japan Advanced Institute Science and Technology)

6.3 OIST-IRP Internal Seminar

Date: December 19, 2008
Venue: IRP Lounge, OIST, Okinawa, Japan
Speaker: Mayuko Hamada (OIST)

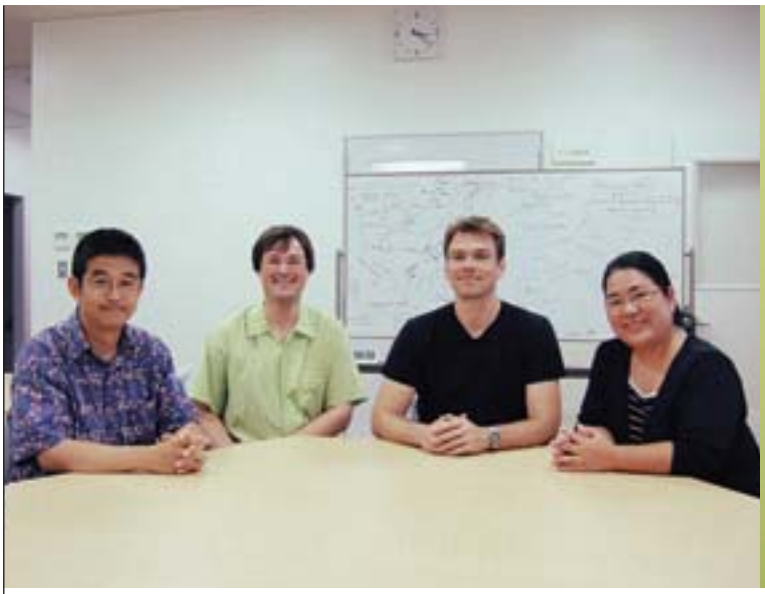
6.4 BioHackathon 2009

Date: March 15–21, 2009
Venue: Database Center for Life Science (DBCLS), Tokyo, Japan
OIST Seaside House, Okinawa, Japan
Co-organizers: Toshihisa Takagi (DBCLS)
Yoshiki Hotta (ROIS)
Nori Satoh (OIST)
Co-sponsors: Database Center for Life Science (DBCLS)
OIST

6.5 Seminar

Date: March 19, 2008
Venue: IRP Conference Room, OIST, Okinawa, Japan
Co-organizer: Takeshi Kawashima (OIST)
Speaker: Nicholas H Putnam (Rice University)

Mathematical Biology Unit



Principal Investigator:
Robert Sinclair

Research Theme:
Mathematical Biology

Abstract

The diversity of life on earth, both what we now observe and what has lived before, is a result of interplay between unchanging physical laws and changing environmental and ecological factors. It is theoretically challenging to attempt to find principles which can help us to understand this diversity or, on another level, analyse experimental data in a meaningful way. Our long-term goal is to extract new mathematics from biology, given that mathematics did grow as a result of contact with physics. This can only be achieved by seriously engaging with biological research at all levels, and OIST is one of the few places in the world where this is in fact a part of the institutional culture. We continue to benefit from lively interactions with other units.

1 Staff

Mathematical Biology Unit

Researchers: Dr. Masahiro Hamano
Dr. Gunnar Wilken

Research Administrator / Secretary: Mrs. Shino Fibbs

2 Partner Organizations

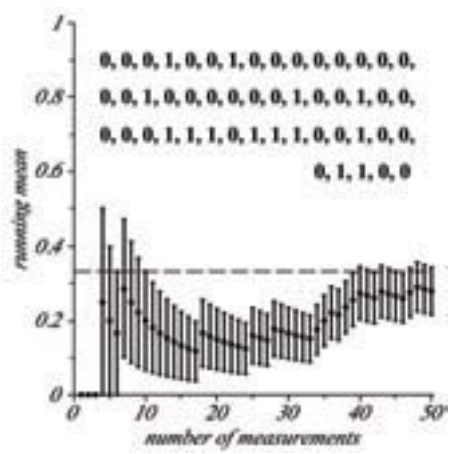
3 Activities and Findings

With the arrival of two new members in October 2008, our research has expanded in scope and energy.

3.1 "Data Analysis – Extremely Small Sample Sizes" (R. Sinclair)

This work has continued to be a major focus of our research efforts. The goal is to generalize the standard error of the mean (S.E.M.), a very commonly used estimate of error in all areas of biology, to cases in which the number of samples is extremely small (less than ten, for example). The goal is to contribute to biological research by producing an error estimate which is reliable and well-motivated. In other words, this work is intended to be a contribution to experimental biology.

It has been possible to formulate a continuity property which any reasonable error estimate should satisfy. This property has allowed us to almost uniquely define an error estimate. This is a significant



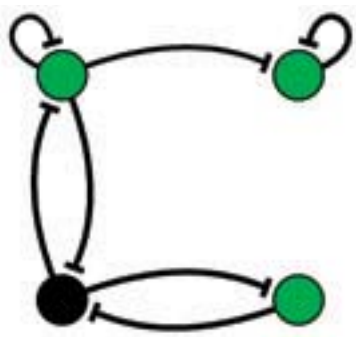
theoretical advance for the project which we believe will find broader application.
During 2008, we have been verifying the hypotheses we have made concerning the exact properties of the error estimate we have constructed. This has involved intensive computation, making use of interval arithmetic. The point of these computations is to prove that the new error estimate actually has a confidence coefficient of 95%. In other words, we are making sure that the new estimate will in fact be useful.

3.2 "Protein Sequence Analysis" (R. Sinclair)

Our focus here is on analytical techniques, both of qualitative and quantitative type, which would allow one to infer the past history of a protein. One question relates to whether a protein which exhibits an imperfect tandem repeat structure has this as a result of a duplication event, or selective pressure to become periodic. A second question is whether a given nuclear plant gene was introduced in an endosymbiotic event or not.
One combinatorial problem which arises is to find equivalent codings for highly conserved amino acids which preserve GC content and dinucleotide counts. Such equivalent codings could become the basis for a type of phylogenetic marker which is independent of many types of selection. It will be necessary to validate any such idea using public sequence databases. This can be seen as a generalization of the use of the two serine coding types to infer lines of descent.
Further, we have devised methods for visualizing relationships between protein (or genomic) sequences without requiring a phylogenetic tree as input. We believe that such visualizations will be very useful in the analysis of sequences for the very fact that they are complementary to standard approaches.
Finally, we are developing qualitative methods for the detection of imperfect tandem repeats which are the result of a duplication event. The central point is to look for amino acid positions which are (apparently) not under selection, since they may lose their phylogenetic memory more slowly than others.

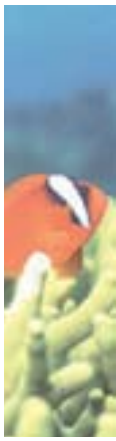
3.3 "Relationships between Complexity and Modularity" (R. Sinclair)

This is recent work which has already produced results by combining abstract formulations and computational proofs. What we have been able to show is that selection for complexity can induce modularity in a synchronized inhibitory boolean network model. The result is theoretically surprising in itself. The model arose from discussions of the complexity of miRNA networks. The computational result is of relevance to a much wider class of questions which arise in discussions of the evolution of complex organisms.



3.4 "Computations in Biology" (M. Hamano)

We are trying to extract computational structures from biological phenomena by using mathematical theories of categories, logics and topologies. To do this, we also consider algebras and computational languages to faithfully describe interactions of genes, proteins and cells. This enables us to apply mathematically rigorous analysis to biological systems, especially with regard to the computational complexity which a biological interaction embodies.
Currently, we are working interpretations of RNA interference in the framework of pi-calculus. The pi-calculus of R.Milner (90's) is a mathematical model of concurrent computation, which is powerful even in comparison with the lambda calculus (a functional model for computing) and Turing machines. In the last decade, owing to its treatment of mobility, the pi-calculus has turned out to be suitable for modelling some biomolecular interactions such as signal transduction pathways and combinatorics of proteins, etc. (work done independently by E.Shapiro, L.Cardelli and V.Danos et al).
Capturing RNAi as a computational structure, our interpretation aims to extract the computational meaning of RNAi, especially its complexity. The interpretation is also expected to provide a mathematically uniform treatment of amplification and feedback in biological networks.
We are also interested in applying these methodologies to (larger) dynamical systems to clarify how emergent properties arise from local interactions of agents.



3.5 "Linear Logic and denotational semantics" (M. Hamano)

Ideas and methods of the representation theory of groups adapt very well to model duality, which is a cornerstone of Linear Logic and provide its denotational semantics. A denotational semantics models computations by certain mathematical objects (e.g., fix-points) invariant under computations. We observe that characters of group representations provide a bridge to a semantics of computability (weaker than computation itself) of a variant of the indexed logic of (Hamano et al, An Indexed System for Multiplicative Additive Polarized Linear Logic, LNCS 5213 Springer-Verlag 262-277, 2008). We are now investigating a completeness theorem of the variant of indexed logic.

3.6 "Discrete State Analysis of Spatial Representation and Memory" (G. Wilken)

Rapidly accumulating experimental and theoretical work in neuroscience suggests that the brain realizes cognitive functions, e.g. spatial representation and memory, by precisely timed oscillatory interaction of neuronal circuits involving multiple local ensembles of various both cortical and subcortical areas. We particularly study models which integrate the phenomena of phase precession and adult neurogenesis within the hippocampal formation, focusing on the dentate gyrus. The recently discovered grid cells in the entorhinal cortex have given rise to numerous competing models, and several scientists have asked for a more general interpretation of existing data addressing the representation of episodes. We investigate the consistency and interplay of models for subunits of functional assemblies involving different brain parts. In a first step we extract quantitative anatomical and physiological data as well as mechanisms of mutual reaction formulated in terms of discrete states. In order to analyze this information we then employ and adapt methods from discrete mathematics, mathematical logic, and theoretical computer science such as elementary combinatorics, graph theory, temporal logics and model checking. Our goal is to contribute to more integrative and coherent modeling on the basis of mathematical reasoning and to gain more understanding of how the brain realizes cognitive functions.
This project started in October 2008.

3.7 "Complexity of Higher Order Rewrite Systems" (G. Wilken)

Lindenmayer systems, which have numerous applications in biological modeling, form a subfamily of rewrite systems whose theory is part of mathematical logic and computer science. A prototype of expressive higher order rewrite systems is Goedel's T formulated in typed lambda-calculus. A detailed analysis of its complexity is joint work with Prof. Andreas Weiermann at the University of Ghent, Belgium, and was recently accepted for publication in Lecture Notes in Computer Science. Another joint article with Prof. Weiermann (on ordinal arithmetic) was submitted in January.

3.8 "Elementary Patterns of Resemblance" (G. Wilken)

This project has its origin in the area of proof theory and is joint work with Prof. Timothy J. Carlson at the Ohio State University, USA. Patterns of resemblance (Carlson, 2001) provide a new approach to ordinal notation systems which in turn open the road for proofs of consistency of mathematical theories and the measurement and hence comparison of their strengths. Patterns are finite structures consisting of nested trees evolving along complex sequences of intriguing self-resembling repetition. An article with Carlson is being finalized for submission. Further articles are in preparation.

4 Publications

4.1 Journals

Bonnard, B., Caillau, J.B., Sinclair, R., & Tanaka, M. Conjugate and cut loci of a two-sphere of revolution with application to optimal control. *Annales de l'Institut Henri Poincare (C) Non Linear Analysis* (2008).

4.2 Book(s) and other one-time publications

4.3 Oral presentations

Sinclair, R. Doing Mathematical Research with Computational Tools: Closed Asymptotic Curves Mathematics and Computers, TMU, Minami-Ohsawa, Japan, May 23, 2008

Wilken, G. Aspekte der Arbeitsweise des Hippocampus, Seminar in Electrical Engineering, TU

Braunschweig, Germany, December 19, 2008

Wilken, G. Proof-theoretic Ordinals arising from Elementary Patterns of Resemblance, Seminar in Logic, Kobe University, Japan, February 22, 2009

Hamano, M. An Indexed System for Multiplicative Additive Polarized Linear Logic, Seminar, PPS, Universite Paris Diderot, Paris 7, France, March 3, 2009

Wilken, G. Tracking Chains for Elementary Patterns of Resemblance, Kansai Set Theory Seminar, Kobe University, Japan, March 5, 2009

4.4 Posters

Torben-Nielsen, B., Sinclair, R., Stiefel, K. M. Synaptic integration involves synapses and dendritic trees. CNS 2008, Portland, Oregon, USA, July 19 - 24, 2008

Torben-Nielsen, B., Sinclair, R., Stiefel, K. M. Fly VS neurons compared to optimized motion detectors. COSYNE 2009, Salt Lake City, USA, February 26 - March 2, 2009

5 Intellectual Property Rights and Other Specific Achievements

Nothing to be reported.

6 Meetings and Events

6.1 Multi-Scale Phenomena in Biology (Workshop)

Date: November 4-6, 2008
Venue: OIST Seaside House
Co-organizer: Klaus Stiefel (OIST)
Speakers: Bjorn Engquist, The University of Texas at Austin
Keiko Takahashi, Earth Simulator Center
Diego Rasskin-Gutman, University of Valencia
Maddalena Venturoli, New York University
Walter Tschinkel, Florida State University
Brian Kinlan, University of California, Santa Barbara
Radek Erban, University of Oxford
Anthony Bell, University of California, Berkeley
Klaus Stiefel, OIST
Werner Callebaut, Konrad Lorenz Institute
Other remarks: In addition to the speakers, 16 participants from 8 countries attended.

6.2 Applications of Integrable Systems Methods to Problems in Geometry (Seminar)

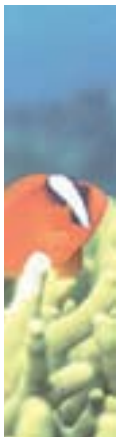
Date: May 28, 2008
Venue: University of the Ryukyus, Rikei-Fukugoto
Co-organizers: Mathematical Sciences, University of the Ryukus
Speaker: David Brander, Kobe University
Other remarks: Joint Mathematics Seminar

6.3 Introduction to the Nervous Systems of Nematodes (Talk)

Date: December 7, 2008
Venue: OITC Annex
Speaker: Prof. Tony Stretton

6.4 Harmonic Maps and the Beauty of Diamond (Talk)

Date: January 29, 2009
Venue: OITC Annex, Uruma
Speaker: Prof. Reiko Miyaoka, Tohoku University



6.5 Circuits, Differentiation and Homeostasis in Genetic Regulatory Networks (Seminar)

Date: February 4, 2009
Venue: IRP Conference Room
Speaker: Paul Ruet, CNRS, France.

Theoretical and Experimental Neurobiology Unit

Principal Investigator:
Dr. Klaus M. Stiefel

Research Theme:
Single cell computation,
Dendritic morphology,
Theoretical Neurobiology

Abstract

The goal of this research unit is to shed more light on the function of individual brain cells. For that purpose, we are using a combination of experimental and theoretical approaches.
The experimental approaches are whole-cell patch clamp recordings in slices of the mouse frontal cortex. The basic question we want to answer is "what type of computer is a neuron"? We are studying several aspects of the signal processing of neurons, such as their precision, phase-reset curves as well as their behavior under intrinsic and forced oscillations. As neurons in the cortex are subject to a variety of neuromodulatory (dopaminergic, cholinergic) influences, we are also studying all the afromentioned phenomena in cells subjected to these modulators.
The theoretical approaches are biophysical simulations of neurons and the use of genetic algorithms.
This year, Maxence LeVasseur left us to start his doctoral study at the University of British Columbia, Canada, and Dr. Marylka Uusisaari joined, coming us from Riken. Dr. Benjamin Torben-Nielsen completed his doctorate with a public exam at the University of Tilburg, the Netherlands (with Dr. Stiefel as a committee member).

1 Staff

Researchers: Dr. Yoe Marylka Uusisaari
Dr. Benjamin Torben-Nielsen
Research Administrator / Secretary: Ms. Ryoko Uchida

2 Partner Organizations

Ecole Normale Supérieure (ENS), Paris, France
Type of partnership: Scientific collaboration
Name of principal researcher: Dr. Boris Gutkin
Research theme: Theory of Neural Spiking

Case Western University, Cleveland, USA
Type of partnership: Scientific collaboration
Name of principal researcher: Prof. Peter J. Thomas
Research theme: Sub-threshold oscillations

University of Sydney, Australia
Type of partnership: Scientific collaboration
Name of principal researcher: Prof. Dario Protti
Research theme: Dynamic clamp recordings in the retina

3 Activities and Findings

This year we continued our investigation into input-output relationships of single cortical neurons.
In our experimental work (in vitro recordings of cortical neurons), we researched the influence of dopamine, serotonin and acetylcholine, three important neuromodulators (substances influencing the basic operating mode of the brain) on neural input-output transformations. Neural features related to these input-output transformations we are interested in are membrane resonance, spiking reliability and precision and phase-reset curves. The data we acquired are currently being analyzed. We also started an experimental project looking at stochastic resonance, the phenomenon that, under certain conditions, information transmission in neurons works best in the presence of a finite amount of noise.

In our theoretical work, we continued to look at how dendritic morphologies optimized for certain computational tasks resemble real neurons. Specifically, we investigated input-order detection and wide-field motion detection, the later a function performed by the well-characterized fly lobular plate neurons. This work is published and under preparation for publication.
Furthermore, we started working with multi-scale models, which integrate the network and the detailed cellular level of neural modeling at one, continuous numerical simulation.

4 Publications

4.1 Journals

Stiefel, K.M., Gutkin, B.S., & Sejnowski, T.J. Cholinergic neuromodulation changes phase response curve shape and type in cortical pyramidal neurons. *PLoS ONE* 3 (12), e3947 (2008).

Stiefel, K.M., Gutkin, B.S., & Sejnowski, T.J. The effects of cholinergic neuromodulation on neuronal phase-response curves of modeled cortical neurons. *J Comput Neurosci* 26 (2), 289-301 (2009).

Torben-Nielsen, B. Dendritic morphology: function shapes structure. (2008). Doctoral Thesis, University of Tilburg.

Torben-Nielsen, B., Vanderlooy, S., & Postma, E.O. Non-parametric algorithmic generation of neuronal morphologies. *Neuroinformatics* 6 (4), 257-277 (2008).

4.2 Book(s) and other one-time publications

Torben-Nielsen, B., Stiefel, K.M. Multi-scale modeling of cortical neural networks *Proceedings of the OIST workshop "Multi-scale pehnomena in biology",* American Institute of Physics 12, 345-678, 2009

4.3 Oral presentations

Torben-Nielsen, B. Function shapes structure: Results from optimizing model neurons for performing particular functions. Redwood Center for Neuroscience, Berkely, CA, USA, March 6, 2009

Torben-Nielsen, B. Investigating the computational relevance of dendrites. OIST internal seminar, Okinawa, Japan, January 16, 2009

Torben-Nielsen, B. Fake real neurons as next generation robot controller? Single-neuron Computation workshop.Tilburg University, the Netherlands, December 2, 2008

Torben-Nielsen, B. A journey along synthetic/fake neurons. Almende, Rotterdam, the Netherlands November 28, 2008

Torben-Nielsen, B. Fake real neurons for robust robot controllers. Interactive Collaborative Information Systems (ICIS) meeting. Delft, the Netherlands, November 24, 2008

Torben-Nielsen, B. Single neurons a rush through four selected topics. OIST Junior Retreat, Okinawa, Japan, October 28, 2008

Stiefel, K. M. Dendritic function-structure mapping Technical University of Graz, Austria, July 2, 2008

Stiefel, K. M. Dendritic function-structure mapping University of Sydney, Australia, December 19, 2008

Stiefel, K. M. Single Neuron Computation University of Brisbane, Australia, March 14, 2008

4.4 Posters

Torben-Nielsen, B., Stiefel, K.M. Fly VS neurons compared to optimized motion detectors. COSYNE 2009, Salt Lake City, February 26, 2009

Torben-Nielsen, B., Stiefel, K.M. Synaptic integration involves synapses and dendritic trees: Results from optimizing neuron models for spatio-temporal integration. CNS 2008, Portland, July 19, 2008

Le Vasseur, M., Stiefel, K. M. Neuromodulation of spiking in the mouse prefrontal cortex. FENS 2008, July 12, 2008

5 Intellectual Property Rights and Other Specific Achievements

6 Meetings and Events

6.1 Single-neuron Computation workshop

Date: December 2nd, 2008
Venue: University of Tilburg
Co-organizer: University of Tilburg
Co-sponsor: University of Tilburg
Speakers: Jaap van Pelt (Free University Amsterdam)
Benjamin Torben-Nielsen (OIST)
Klaus M. Stiefel (OIST)
Erik Postma (University of Tilburg)

6.2 Okinawa Computational Neuroscience Course

Date: June 16th - July 4th 2008
Venue: OIST Seaside House
Co-organizers: Drs. Erick Deshutter, Kenji Doya, and Jeff Wickens (OIST)
Speakers: Arbuthnott, Gordon (OIST)
Bell, Anthony (UC Berkeley)
Bhalla, Upi (Tata Institute of Fundamental Research, India)
Butera, Robert (Georgia Institute of Technology)
Deneve, Sophie (ENS, Paris)
De Schutter, Erik (OIST)
Destexhe, Alain (UNIC, France)
Doya, Kenji (OIST)
Fairhall, Adrienne (Weizmann Institute of Science)
Gewaltig, Marc-Oliver (Bernstein Center for Computational Neuroscience)
Häusser, Michael (University College London)
Ishii, Shin (NAIST)
Koch, Christof (California Institute of Technology)
Li, Zhaoping (University College London)
Longtin, André (Ottawa Institute of Systems Biology)
Stiefel, Klaus (OIST)
Tsodyks, Misha (Weizmann Institute of Science)
Wang, Xiao-Jing (Yale University School of Medicine)
Wickens, Jeff (OIST)

6.3 Multi-Scale Phenomena In Biology

Date: November 4th – 6th, 2008
Venue: OIST, Seaside House
Co-organizer: Robert Sinclair
Speakers: Bjorn Engquist (The University of Texas at Austin)
Hans Othmer (University of Minnesota)
Keiko Takahashi (Earth Simulator Center)
Diego Rasskin-Gutman (University of Valencia)
Klaus Stiefel (OIST)
Anthony Bell (UC Berkeley)
Robert Warner (University of California Santa Barbara)
Walter R. Tschinkel (Florida State University)
Werner Callebaut (Konrad Lorenz Institute)
Maddalena Venturoli (Courant Institute for Mathematical Science)

6.4 Talk for Junior High School Students

Date: February 18, 2008
Venue: Nakadomari Junior High School, Onna
Co-organizer: OIST Community Relations Office
Speaker: Klaus M. Stiefel (OIST)

6.5 OIST Open House 2008

Date: November 9, 2008
Venue: Okinawa Industrial Technology Center (OITC)
Co-organizers: Klaus Stiefel, Mary Ann Price, Gail Tripp and the Community Relation Office at OIST
URL: http://www.oist.jp/j/doc/20081109openhouse_rev.pdf

Cellular & Molecular Synaptic Function Unit

Principal Investigator:
Tomoyuki Takahashi

Research Theme:
Regulatory mechanisms for transmitter release

Abstract

In the neuronal system, dynamic changes of synaptic strength play critical roles in switching neuronal circuits. Regarding synaptic strength, compared with postsynaptic mechanisms much less is known for presynaptic mechanisms, primarily because of small nerve terminal structures preventing applications of electrophysiological and imaging techniques. The calyx of Held is a giant glutamatergic nerve terminal visually identified in the mammalian auditory brainstem slices. By applying molecular, imaging and patch-clamp techniques to the synapse of developing rodents, we aim at elucidating regulatory mechanisms of transmitter release.

The calyx of Held synapse undergoes rapid developmental changes in structure, functional properties and molecular compositions during the second postnatal (P) week, across the hearing onset (P10-12). Our progress in the fiscal year 2008 is as follows.

(1) The Ca/calmodulin (CaM)-dependent mechanism underlay short-term synaptic plasticity as well as synaptic vesicle enodocytosis. However, the mechanism underwent a developmental decline, despite persistent expression of CaM in the calyx terminal. Confocal spot Ca imagings at the calyx terminal revealed that the Ca domain (comprising synaptic vesicles and Ca channels) underwent a developmental segregation and confinement toward tight Ca-secretion couplings. This developmental change minimizes bulk residual Ca accumulation, thereby downregulating the CaM activity, which requires relatively high Ca concentrations.

(2) Transmitter glutamate released from synaptic vesicles can activate and desensitize postsynaptic AMPA receptors (AMPAR). From the effects of the AMPAR desensitization blocker CTZ on EPSCs, it is suggested that AMPAR desensitization is involved in the short-term synaptic depression, as well as in saturation of synaptic transmission at high release probability, at calyceal synapses before the hearing onset, but no longer after hearing. Quantitative PCR data on tissues at the postsynaptic region, and immunocyto-chemical data on postsynaptic cells, suggested that this developmental change is caused in part by a developmental decline in the AMPAR subunit GluR1. Manipulation of transmitter release probability suggested that high release probability at prehearing calyces is also responsible. Thus, both pre and postsynaptic factors underlie the developmental decline in the involvement of desensitization in the synaptic efficacy.

(3) The mechanism by which presynaptic depolarization affect transmitter release remains open. In simultaneous pre- and postsynaptic patch-clamp recordings at the calyx of Held, we clarified that

(i) Ca/NCS-1-dependent facilitation of presynaptic Ca channel gatings contributes to presynaptic facilitations by roughly 50% under various intraterminal Ca buffer conditions.

(ii) Complete block of facilitation required intra-terminal presence of fast-binding buffer BAPTA, suggesting that facilitation mechanism of transmitter release resides in the immediate vicinity of Ca channels.

1 Staff

Cellular & Molecular Synaptic Function Unit

Researchers: Takayuki Yamashita,
Kogaku Eguchi,
Hiroyasu Watanabe,
Tetsuya Hori,
Yukihiro Nakamura

Technical Staff: Shoko Motohashi

Research Administrator / Secretary: Kaori Egashira

2 Partner Organizations

Doshisha University Faculty of Life and Medical Sciences

Type of partnership: Joint research
Name of principal researcher: Tomoyuki Takahashi
Name of researcher: Naoto Saitoh
Research theme: Regulatory mechanisms for transmitter release

Kyoto University Faculty of Engineering, Department of Biological Chemistry and Molecular Biology

Type of partnership: Scientific collaboration
Name of principal researcher: Yasuo Mori
Name of researchers: Yasuo Mori, Shigeki Kiyonaka
Research theme: Presynaptic roles of Ca binding proteins

University Paris V

Type of partnership: Scientific Collaboration
Name of principal researcher: David DiGregorio
Name of researcher: David DiGregorio
Research theme: Developmental changes in presynaptic Ca signal profiles associated with transmitter release

Vollum Institute, Oregon Health & Science University

Type of partnership: Scientific Collaboration
Name of principal researcher: Henrique von Gersdorff
Name of researcher: Henrique von Gersdorff
Research theme: Developmental changes in the mechanisms underlying synaptic vesicle endocytosis

National Institute for Physiological Sciences, Department of Cerebral Research, Division of Cellular Structure

Type of partnership: Scientific Collaboration
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Research theme: Developmental changes in the presynaptic Ca binding proteins

3 Activities and Findings

3.1 Developmental changes in presynaptic mechanism underlying short-term synaptic depression

Presynaptic Ca channels play pivotal role in triggering transmitter release. Upon repetitive or prolonged activation, presynaptic Ca channels undergo inactivation, thereby contributing to short-term depression of synaptic transmission. At the immature calyx of Held, before the hearing onset, Ca and calmodulin (CaM) play essential roles in Ca channel inactivation, as assessed from a block of inactivation by CaM inhibitors (Fig 1). As animals mature and start to hear sounds, however, the CaM inhibitor no longer affected Ca channel inactivation, or short-term synaptic depression.

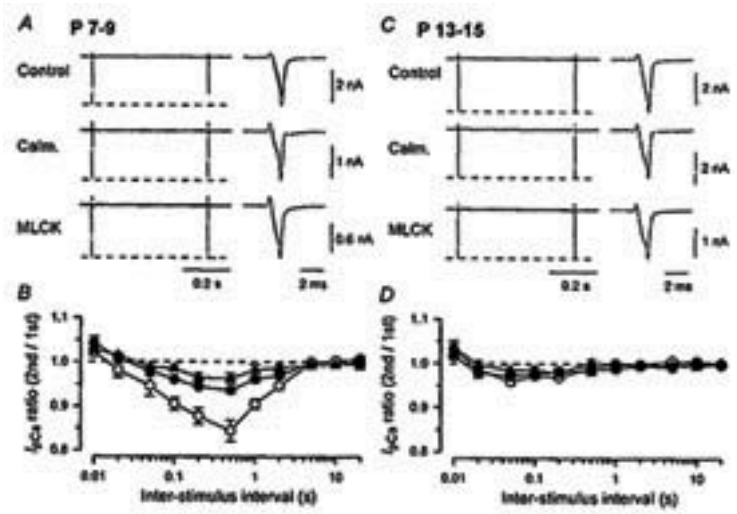

Fig 1 Developmental changes in presynaptic Ca current inactivation. B & D, ratio of the second Ca current amplitude relative to the first one (ordinates) in paired pulse protocol at different inter-pulse intervals (abscissae). Filled symbols indicate data in the presence of CaM inhibitors MLCK peptide and calmidazolium, both of which attenuated Ca current inactivation peaked at 0.5 s interval at P7-9 rat calyces, but no CaM-dependent inactivation was observed at P13-15 calyces.

This developmental change was not caused by that of CaM, as it was expressed in calyx terminals to a similar extent throughout the second postnatal week (Fig 2).

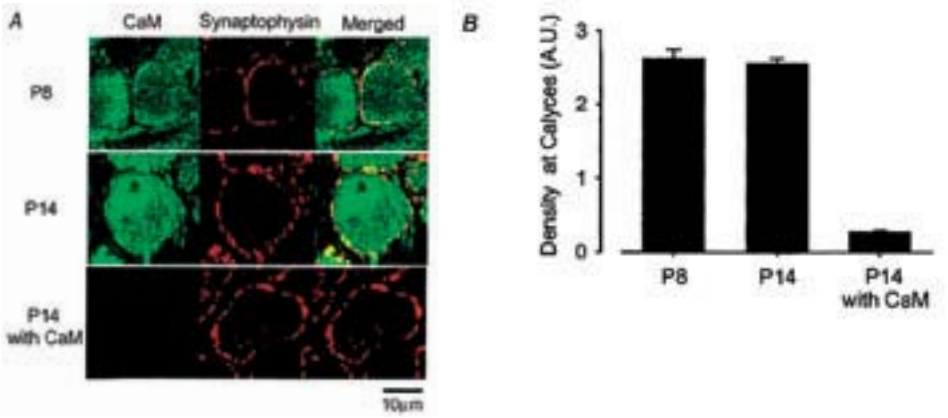

Fig 2 CaM immunoreactivities at P8 and P14 calyces.

At calyces in the post-hearing period, tetanic activation of Ca channels using action-potential waveform command voltage pulses at 500 Hz caused their facilitation and inactivation, both of which could be attenuated by intraterminal injection of a CaM inhibitor peptide (Fig 3).

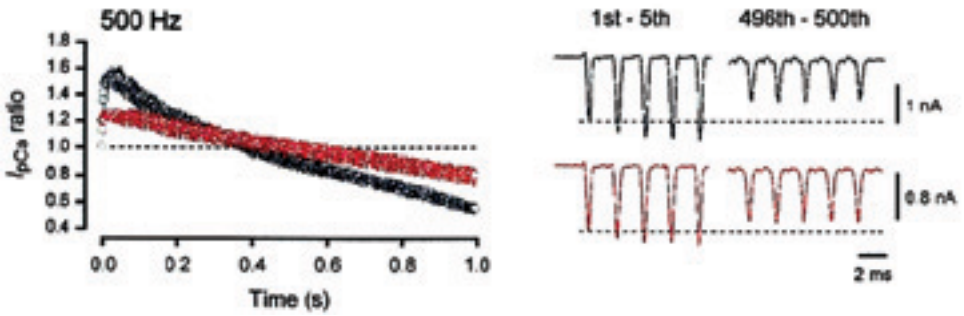

Fig 3 Involvement of CaM in activity-dependent inactivation of presynaptic Ca currents at P13-15 calyces. Ca currents were elicited by action potential waveform command pulses in the presence (red) or absence (black) of the CaM inhibitor MLCK peptide.

These results suggest that upon repetitive stimulation intraterminal Ca concentration can accumulate to a high level, above the activation threshold of CaM, at immature synapses, whereas Ca

accumulation becomes less as animals mature. Thus the Ca/CaM dependent mechanism underlies synaptic depression only at prehearing synapses, and this mechanism becomes inoperative as intraterminal Ca concentration dynamics is refined for tight Ca-secretion coupling (Nakamura T *et al,* 2008).

3.2 Developmental changes in the mechanisms underlying synaptic vesicle endocytosis

After exocytotic release of transmitter, synaptic vesicles, fused into the plasma membrane, are retrieved by endocytosis into the nerve terminal. These endocytotic changes can be monitored by membrane capacitance measurements at the calyx of Held (eg, Yamashita et al, *Science* 2005). During tetanic stimulation applied to the nerve terminal, endocytotic membrane changes following individual exocytosis become faster, and their peaks sum up. At prehearing calyces, this accelerated endocytosis could be dissected into a Ca/CaM dependent and –independent components, the former of which was also GTP-dependent, whereas the latter was GTP-independent, as deduced from a partial block of endocytosis by the non-hydrolysable GTPγS at a high concentration (5 mM). As rats matured, vesicle endocytosis became entirely GTP-dependent, and Ca/CaM-dependent endocytosis was no longer observed. These results are in line with those on the Ca channel inactivation mechanism (see above 3.1), suggesting that the presynaptic modulatory role of CaM becomes insignificant during early postnatal development (Yamashita *et al,* in preparation).

3.3 Developmental changes in the intraterminal profile of Ca entry

By applying the confocal spot Ca detection method to the calyx of Held, we recorded fast Ca transients in response to a single action potential (Fig 4).

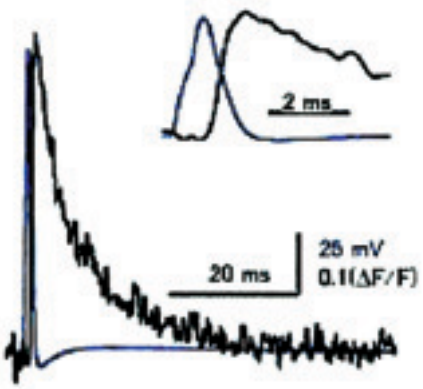

Fig 4 An averaged Ca transient (black traces) elicited at a confocal spot in a calyceal nerve terminal in response to a presynaptic action potential (superimposed blue traces), at two different time scales.

At prehearing calyces, these transients could unfailingly be observed along the edge of the terminal in apposition to the postsynaptic cell (Fig 5).

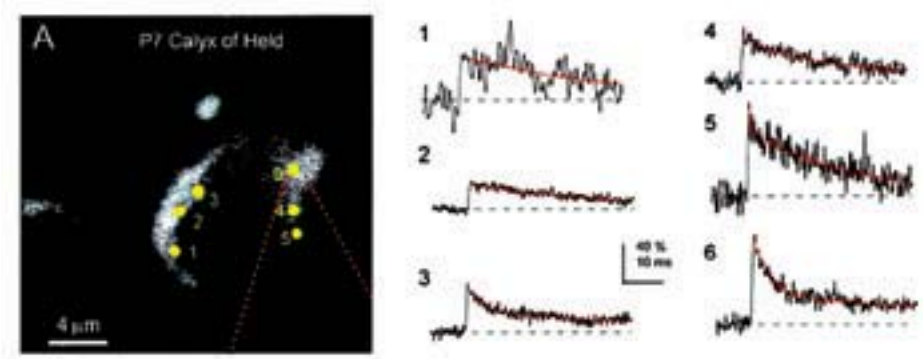

Fig 5 Ca transients at different locations (1-6, yellow dots) elicited by single action potentials in a calyceal presynaptic terminal loaded with 100 μM Oregon green BAPTA-5N for Ca measurements and Alexa 647 for terminal visualization.

As animals start to hear, however, hot spots of Ca transients became less frequent and their amplitudes became smaller, despite the fact that their rise-time kinetics were faster compared with those in preheraing calyces. Given that total Ca currents evoked by whole terminal stimulation remains unchanged during the development, these results suggest that Ca channels in calyceal

terminals undergo developmental decrease in their density. Such a developmental reformation will shorten the Ca-secretion coupling distance, thereby establishing fast efficient transmitter release required for high-fidelity synaptic transmission. This developmental change would also reduce accumulation of residual Ca after entry, thereby eliminating Ca-dependent modulatory functions for transmitter release, mediated by low-affinity Ca binding proteins such as CaM (Nakamura Y *et al,* in preparation).

3.4 Developmental changes in the involvement of AMPAR desensitization in the short-term synaptic depression

Postsynaptic AMPA receptors (AMPARs) undergo desensitization during repetitive activation by the transmitter glutamate. The recovery time course from desensitization, assessed from paired-pulse rapid glutamate applications to excised patches of postsynaptic MNTB neuronal membrane, had double exponential components with a weighted mean time constant of 150 ms at postnatal day (P) 7. This time course became faster and mono-exponential as rats matured, to be 27 ms at P14 and 16 ms at P21 (Fig 6). AMPAR-mediated synaptic currents (EPSCs) evoked at the calyx of Held showed paired-pulse depressions (PPD) at different inter-stimulus-intervals up to 1s. Cyclothiazide (CTZ, 0.1 mM), which can abolish AMPAR desensitization, attenuated the PPD at prehearing (P7) synapse, but had no effect on PPD at posthearing synapses (P14) (Fig 7).

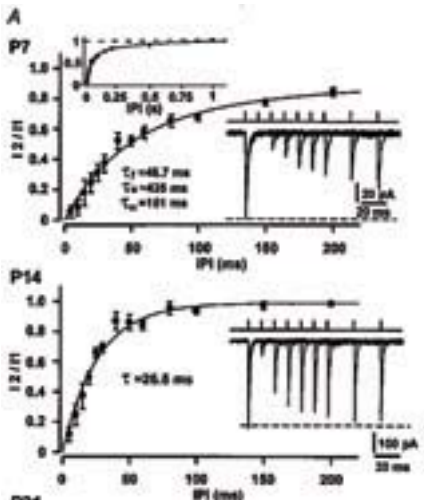

Fig 6

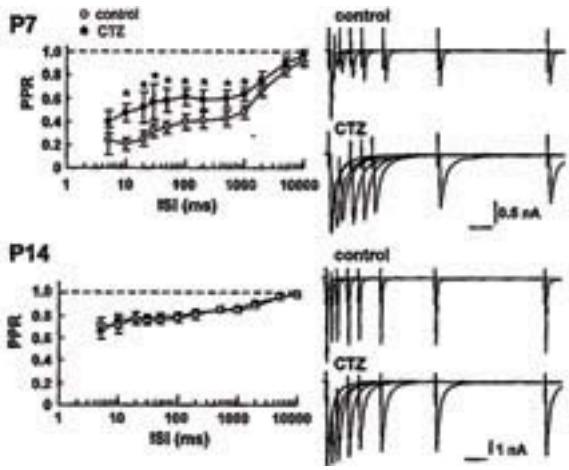

Fig 7

Fig 6 Recovery time course of AMPARs from desensitization at P7 and P14. Paired-pulse glutamate application to outside-out patches excised from MNTB neurons. AMPAR patch currents are shown in the right insets (superimposed).

Fig 7 Paired pulse synaptic depression at different interpulse intervals (ISI, abscissae) in the presence (filled circles) or absence (open circles) of CTZ. EPSCs are shown (superimposed) on the right panels.

Quantititive RT-PCR and immunocyto chemical analyses (Fig 8) indicated that GluR1 subunit AMPARs in MNTB neurons undergo developmental decline.

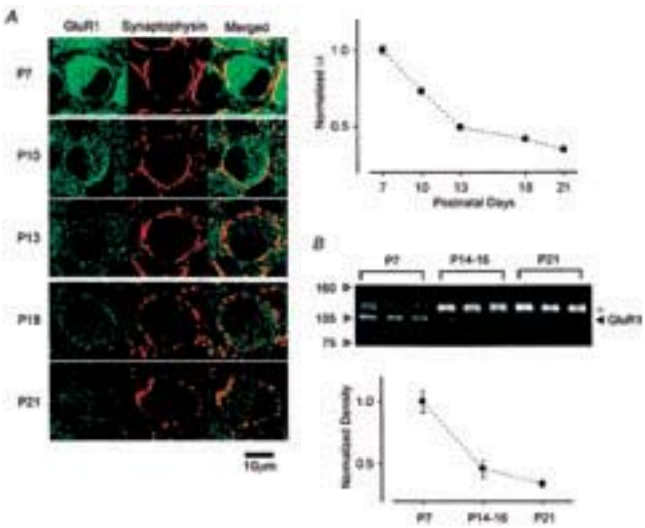

Fig 8 Developmental decline in the GluR1 immunoreactivity (i.r.) in MNTB neurons. A, i.r. pictures of GluR1 with synaptophysin for a presynaptic marker. Right panel shows developmental decline in the densitometrically quantified GluR1i.r. B, Western blot of GluR1 for the tissue from the MNTB region of developing rats.

These results are consistent with previous reports that recombinant AMPARs containing GluR1 have the slowest recovery from desensitization. At the calyx of Held, like at other central synapses, release probability declines during development (Iwasaki & Takahashi, 2001 J Physiol). We tested whether this presynaptic change might also be responsible for developmental decline in the involvement of desensitization in synaptic depression, by manipulating the release probability with different Ca/Mg compositions in bath solution to mimic the developmental change. Reducing the release probability at P7 synapses down to the level at P14 markedly diminished the blocking effect of CTZ on PPD, whereas increasing the release probability at P14 synapses to the P7 level revealed the CTZ-sensitive PPD component (Fig 9).

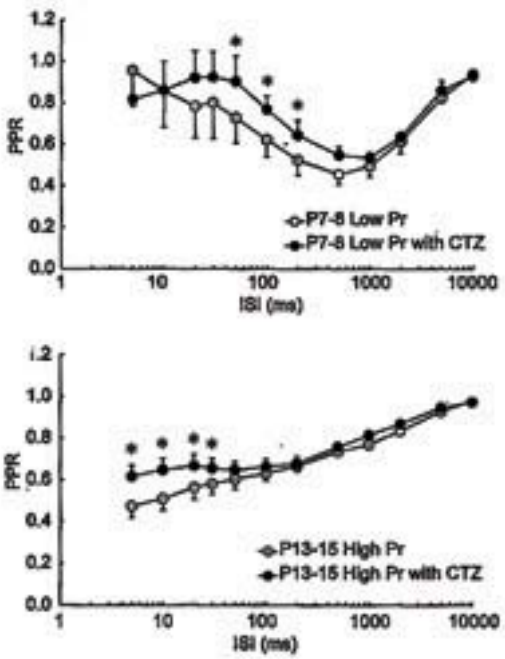

Fig 9 Paired-pulse ratio (PPR; the second/first EPSC amplitude ratio) at different ISI in the presence (filled circles) and absence (open circles) of CTZ at P7-8 calyceal synapses, with their release probability being lowered to match that at P13-15 (upper panel), or raised to match that at P7-8 at P13-15 synapses (lower panel).

Thus, developmental decline in the involvement of AMPAR desensitization in synaptic depression is caused by both postsynaptic (GluR1 upregulation) and presynaptic (decline of release probability) mechanisms (Koike-Tani et al, 2008).

3.5 Involvement of AMPAR desensitization in AMPAR saturation by glutamate released from multiple vesicles

Whether or not vesicular glutamate saturates postsynaptic AMPARs is a fundamental issue for the mechanism of synaptic plasticity as AMPAR saturation precludes synaptic facilitation caused by presynaptic mechanisms. In fact at immature calyces of Held, increasing release probability fails to facilitate EPSCs. Using presynaptic pipette perfusion techniques, in simultaneous pre- and postsynaptic whole-cell recordings, we addressed whether a single vesicle glutamate can saturate postsynaptic AMPARs, and also whether desensitization of AMPARs contributes to their saturation at the calyx of Held in prehearing rats. High concentration of glutamate (up to 100 mM), when loaded into the presynaptic terminal, enhanced the amplitude of spontaneous quantal EPSCs (Fig 10), indicating that glutamate from a single vesicle cannot normally saturate postsynaptic AMPARs at this immature terminal (Yamashita et al, 2009), as we previously reported at more mature terminals (Ishikawa et al, Neuron 2002; Yamashita *et al*, J Neurosci 2003). In contrast, intraterminal loading of glutamate had no effect on the amplitude of nerve-evoked EPSCs suggesting that glutamate released from multiple synaptic vesicles saturate postsynaptic AMPARs.

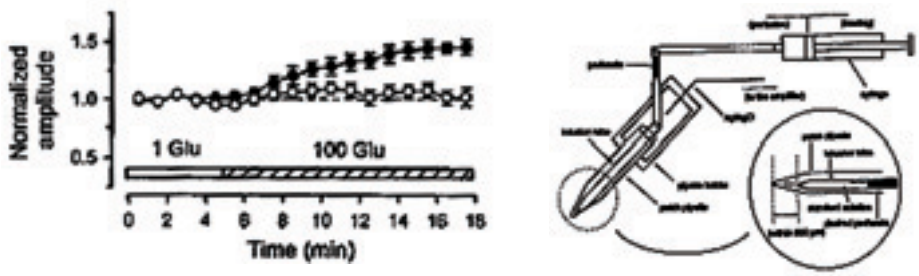

Fig 10 The effect of switching presynaptic L-glutamate concentration from 1 mM to 100 mM by whole-cell pipette perfusion at calyceal terminal on EPSC amplitude. The quantal EPSCs (filled circles) became larger after infusion, whereas nerve-evoked EPSCs (open circles) remained unchanged. Right panel shows a devise used for glutamate infusion.

This saturation could be rescued by reducing release probability by lowering Ca/Mg ratio of artificial cerebrospinal fluid (aCSF), or by simply adding CTZ to aCSF (Fig 11).

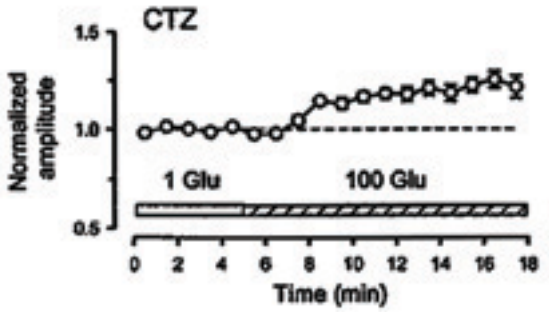

Fig 11 Enhancement of nerve evoked EPSCs by intraterminal infusion of 100 μM L-glutamate in the presence of CTZ.

These results suggest that glutamate released from multiple vesicles at high probability summate to high concentrations in synaptic cleft, thereby desensitizing postsynaptic AMPARs. Thus, desensitization can be involved in saturation of postsynaptic AMPARs by multiple glutamate quanta. As animals mature, release probability declines (Iwasaki & Takahashi, 2001 J Physiol) and recovery from postsynaptic desensitization becomes faster (3.4, Fig 6), resulting in non-saturation of postsynaptic AMPARs by glutamate released by nerve stimulation (Ishikawa et al, 2002 Neuron; Yamashita et al, 2003 J Neurosci).

3.6 Mechanisms underying short-term presynaptic facilitation and depression following presynaptic depolarization

Presynaptic terminals can be depolarized for various lengths of period by a variety of factors. However, the mechanisms by which presynaptic depolarization affects transmitter release remain unestablished. In simultaneous pre- and postsynaptic voltage-clamp recordings at the calyx of Held of mice and rats, moderate presynaptic depolarization increased the amplitude of presynaptic Ca currents and EPSCs (Fig 12).

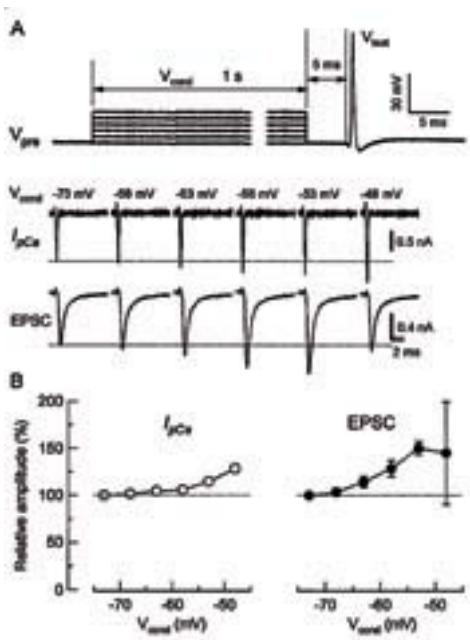

Fig 12 Facilitation of presynaptic Ca currents (I_{pCa}) and EPSCs by sustained presynaptic depolarization (ordinate in B). In simultaneous presynaptic and postsynaptic voltage-clamp recording, EPSCs were elicited by an action potential waveform command pulse (V_{test}) following depolarizing conditioning pulses (V_{cond}, 1s) of different amplitudes (abscissae in B).

Voltage-clamp command pulse cancellation of this Ca current facilitation revealed that it contributes to EPSC facilitation, on average by 50%, in various Ca buffer conditions in the presynaptic terminal (Table 1).

Table 1

	Buffers	EPSC facilitation (%)	I_{pCa} facilitation (%)	I_{pCa} contribution (%)	EPSC amplitude (nA)	n
SDE (-53mV V_{hold}, 1 mM kynurenate)	0.5mM EGTA	154 ± 17	111 ± 2.2	45 ± 6	1.7 ± 0.2	5
	10mM EGTA	123 ± 1.3	105 ± 1.2	34 ± 6	1.3 ± 0.3	4
	0.025mM BAPTA	213 ± 28	120 ± 1.7	67 ± 4	1.3 ± 0.3	4
PPF (5 ms ISI, BoNT/E)	0.1mM EGTA	224 ± 19	127 ± 5.8	40 ± 8	0.8 ± 0.1	5
	0.5mM EGTA	185 ± 7.2	119 ± 1.5	54 ± 9	0.8 ± 0.1	5
	10mM EGTA	176 ± 7.5	116 ± 2.8	48 ± 10	0.4 ± 0.0	4

Activity-dependent synaptic facilitation is thought to arise from residual Ca in the terminal, but EGTA loaded into the terminal at 10 mM only partially attenuated the EPSC facilitation induced by sustained presynaptic depolarization or paired-pulse protocol (Fig 13).

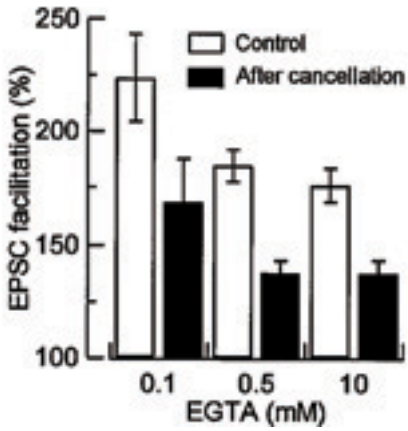

Fig 13 Paired-pulse EPSC facilitations in the presence of EGTA at different concentrations in the calyceal presynaptic terminals. Filled bars represent facilitation components of EPSCs not mediated by Ca current facilitation.

Only after additional loading of the fast Ca-binding buffer BAPTA (1 mM) into the terminal, synaptic facilitation was abolished (Fig 14).

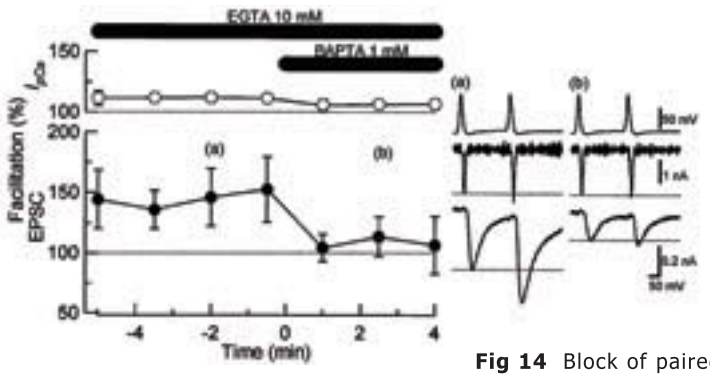

Fig 14 Block of paired-pulse EPSC facilitation by intraterminal perfusion of BAPTA in addition to 10 mM EGTA.

We conclude that the mechanism underlying facilitation of transmitter release resides in the immediate vicinity of Ca channels, where the slow binding Ca buffer EGTA cannot reach in time of Ca entry (Hori & Takahashi, 2009).

3.7 Direct interaction of AP-2 with Ca channel synprint site involved in synaptic vesicle endocytosis

In the affinity column chromatography, we have found that the clathrin-coated endocytosis adaptor protein AP-2, at its μ subunit, directly binds to Ca channel synprint site in II-III loop. This binding was apparently Ca concentration-dependent in brain lysate, in competition to the interaction between synprint and synaptotagmin 1. Intra-terminal loading of synprint site peptide fragment or AP-2 μ fragment, as dominant negative constructs, at the calyx of Held markedly attenuated vesicle endocytosis without affecting exocytosis, as assessed from membrane capacitance changes.

These results suggest that Ca channel synprint site contribute to efficient vesicle endocytososis by interacting with AP-2 and synaptotagmin in a Ca concentration-dependent manner. Thus our results contrast to the "synprint hypothesis" proposing that the synprint site is directly involved in vesicle exocytosis (Watanabe *et al,* submitted).

3.8 Auditory activity-dependent developmental changes in glycinergic inhibitory transmission and glycine receptor subunits

Glycinergic inhibitory transmission in auditory brainstem plays critical roles in high-fidelity auditory transmission, which is required for sound localization. Glycinergic IPSCs recorded from MNTB principal neurons undergo developmental speeding in their decay time during the second postnatal week across the period of hearing onset. To address whether auditory activity plays a role in this developmental change, we performed bilateral cochlear ablation (BCA) in mice at P9, just before the hearing onset (P10-12). In brainstem slices of BCA mice at P14, the weighted mean decay time constant of glycinergic IPSC was similar to that at P7, whereas in sham-opertaed or intact mice at P14, the time constant was signigficantly faster than that at P7. These results suggest that auditory activity is required for developmental speeding of the IPSCs. We next addressed whether these activity-dependent developmental changes occur in glycine receptor subunit compositions in MNTB neurons, by applying quantitative RT-PCR to tissues from the MNTB region of developing mice. During postnatal development, α1 subunit mRNAs increased, whereas α2 subunit mRNAs decreased, as previously reported. In BCA mice, developmental decline in α2 subunit mRNA was similar to sham-operated control mice, but developmental increase in α1 subunit mRNAs was markedly inhibited. Given that recombinant α1 homomeric glycine receptors expressed in *Xenopus* oocytes are characterized with more than 30 times shorter channel life time compared with α2 homomeric glycine receptors (Takahashi et al, *Neuron*, 1992), it is suggested that neuronal activity produced by auditory inputs plays a critical role in the developmental upregulation of glycine receptor α1 subunit, and ensuing speeding of inhibitory synaptic transmission (Ohshima-Takago *et al*, paper in preparation).

3.9 Formation of calyx type synapses in dissociated cell culture

Molecular imagings have been made at a variety of cell structures and have provided essential information on cellular functions. At mammalian central synapses, however, application of imaging techniques is limited to small terminals in culture. To visualize dynamics of synaptic vesicles, and other organellas within the terminal, it is desirable to develop a culture preparation, where large calyceal terminals can be used for imaging studies. To this end, we have recently succeeded in producing a large calyx type synapse in dissociated culture of brainstem neurons. In this preparation, we were able to transfect presynaptic neurons with GFP, and follow axonal growth. After the axon terminal made a contact with a presumptive MNTB neuron, it grew into a large spoon-shaped structure and then transformed to a finger-like structure by "fenenstration", recapitulating *in vivo* development of the calyces of Held. These calyceal synapses were functional as EPSCs could be recorded from postsynaptic neuron in response to presynaptic fiber stimulation. Also, FM-143 dye was taken up specifically by the terminal by endocytosis (Saitoh *et al,* unpublished observations).

4 Publications

4.1 Journals (OIST researchers are underlined)

Hori, T. & Takahashi, T., Mechanisms underlying short-term modulation of transmitter release by presynaptic depolarization. *J Physiol* 587, 2987-3000 (2009).

Yamashita, T., Kanda, T., Eguchi, K., & Takahashi, T. Vesicular glutamate filling and AMPA receptor occupancy at the calyx of Held synapse of immature rats. *J Physiol* 587, 2327-2339 (2009).

Koike-Tani, M., Kanda, T., Saitoh, N., Yamashita, T., & Takahashi, T. Involvement of AMPA receptor desensitization in short-term synaptic depression at the calyx of Held in developing rats. *J Physiol* 586, 2263-2275 (2008).

Nakamura, T., Yamashita, T., Saitoh, N., & Takahashi, T. Developmental changes in calcium/calmodulin-dependent inactivation of calcium currents at the rat calyx of Held. *J Physiol* 586, 2253-2261 (2008).

4.2 Book(s) and other one-time publications

None

4.3 Oral presentations

Hori, T., Sahara, Y., Kaneko, M., Takahashi, T. Maintenance of vesicular glutamate at the calyx of Held presynaptic terminal, The 31st Annual Meeting of the Japan Neuroscience Society, Tokyo, Japan, July 11, 2008

Yamashita, T. Developmental changes in presynaptic functions of calmodulin, OIST-IRP Internal Seminar, Uruma, Japan, August 15, 2008

Yamashita, T., Eguchi, K., Takahashi, T. Developmental change in the mechanism underlying activity-dependent acceleration of sysnaptic vesicle endocytosis, National Institute for Physiological Science Meeting, Okazaki, Japan, September 19, 2008

4.4 Posters

Yamashita, T., Eguchi, K., Takahashi, T. Calmodulin-dependent endocytosis of synaptic vesicles at a fast central synapse, Society for Neuroscience Annual Meeting, Washington D.C., USA, November 17, 2008

Watanabe, H., Yamashita, T., Saitoh, N., Takahashi, T. Direct binding of AP-2 to calcium channel synprint site is essential for clathrin-coated synaptic vesicle endocytsis, Society for Neuroscience Annual Meeting, Washington D.C., USA, November 17, 2008

Yamashita, T., Eguchi, K., Takahashi, T. Ca^{2+}-dependent and independent endocytosis of synaptic vesicles at a fast central synapse, Japan Neuroscience Society Annual Meeting, Tokyo, Japan, July 9, 2009

Watanabe, H., Yamashita, T., Saitoh, N., Takahashi, T. Direct interaction of AP-2 and calcium channel synprint site promotes synaptic vesicle endocytosis, Japan Neuroscience Society Annual Meeting, Tokyo, Japan, July 11, 2009

Hori, T., Takahashi, T. Involvement of presynaptic calcium current facilitation in depolarization-induced facilitation of transmitter release, Society for Neuroscience (Neuroscience 2008), Washington D.C., USA, November 18, 2008

5 Intellectual Property Rights and Other Specific Achievements

None

6 Meetings and Events

6.1 Seminar

Date: June 30, 2008
Venue: IRP
Speaker: Michael Hausser, University College London

6.2 Lecture

Date: October 28, 2008

Venue: Gushikawa Junior high school
Speaker: Tomoyuki Takahashi, OIST

6.3 Seminar

Date: January 23, 2009
Venue: IRP
Speaker: Iori Ito, NIH

6.4 Seminar

Date: February 23, 2009
Venue: IRP
Speaker: Laurent Guillaud, University of Tokyo Graduate School of Medicine

Electron Holography Unit

Principal Investigator:
Akira Tonomura

Research Theme:
Electron Microscope

Abstract

The aim of this project is to understand microscopic behaviors of materials through high-precision electron phase measurement using our coherent electron wave techniques.

Achievements of this year have been made mainly for nanoscale magnetism research using our microscope. The accomplishment includes the observation of magnetic domain structures in writer pole tip for perpendicular recording head. A direct observation of the in-plane domain structure of the writer pole of a perpendicular recording head was performed by electron holography in order to investigate the cause of pole erasure due to the instability of the domain wall behavior.

The instability of domain structure of the writer pole generated a stray field on the air bearing surface of the writer pole. The domain wall trapping at the pole tip was found quite effective to form a stable domain structure in the remanent state, and it reflected domain wall energy change and initial domain structure of the writer pole. Domain wall trapping is effective in obtaining a reversible domain structure, and it relates to the domain wall energy at the writer pole surface.

The domain wall trapping is occurred at the corner of the writer pole that changes the internal stress locally. In particular, domain wall trapping at the flare point can create a closure domain at the throat height in the writer pole tip, which is effective in reducing the remanent magnetization.

1 Staff

Research Advisor: H. Ezawa
Researchers: J.J. Kim, H. Kasai and S. Mamishin
Technical Staff: Y. Tsukada
Research Assistant / Graduate Student: Y. Tamura
Research Administrators / Secretaries: C. Murota and C. Nakamura

2 Partner Organizations

Tohoku University
Type of partnership: Joint Research
Name of principal researcher: D. Shindo
Name of researcher: Y. Murakami
Research theme: Magnetic domain observation in writer pole tip for perpendicular recording head

TDK, Ltd.
Type of partnership: Joint Research

Name of principal researcher: K. Yanagiuchi
Name of researchers: K. Hirata and Y. Ishida
Research theme: Magnetic domain observation in writer pole tip for perpendicular recording head

3 Activities and Findings

3.1 Magnetic domain observation in writer pole tip for perpendicular recording head

Perpendicular recording has an excellent write efficiency compared to longitudinal recording since a medium is magnetized by the strong magnetic coupling between a single writer pole tip in a recording head and the soft under layer of the medium [1–3]. However, this strong magnetic coupling occasionally brings a write instability such as a write-induced signal erasure, i.e., the so-called 'pole erasure' [4]. Thus, understanding the magnetic domain structure in the recording head is central to clarifying and solving the problem of write instability. In this study, we report the observation results that clarify the domain structures of the nanosized pole tips made of Ni-Fe and Co-Ni-Fe by means of electron holography. The change in the domain structures and the detailed magnetic flux distributions inside/outside these pole tips upon the application of magnetic fields of varied strengths were visualized on a nanometer scale. In addition, we report the direct observation result that implies the pole erasure characteristic of the Co-Ni-Fe pole tip.

For this study, Ni-Fe and Co-Ni-Fe writer poles were selected from a variety of writer poles available for recording heads. Each writer pole consists of a pole tip and a rectangular-shaped yoke [Fig. 1]. The magnetic properties measured for thin films of these writer poles are presented in Table 1. It is noted that in the pole erasure testing experiment performed by using a spin stand, pole erasure was observed for the Co-Ni-Fe writer pole, and not for the Ni-Fe writer pole. In order to induce the magnetization process in the specimens, we used a direction-free magnetic field application system [5], installed inside the OIST 300 kV FE-TEM. In the experiments, after applying a magnetic field of 48 kA/m in a direction parallel to that of the electron beam, the strength of the applied magnetic field (H_{appl}) in the specimen plane was controlled by means of tilting operations as shown in Fig. 1 [6]. On the other hand, we utilized an electron holography technique to visualize the domain structure of the pole tip and the magnetic field leaking from it. This technique provides a unique method for detecting the phase shift of the electron beam, from which the electromagnetic field can be evaluated. Therefore, it is expected that we can obtain a magnetic flux distribution in a pole tip on a nanometer scale. The holograms (overlap width: 1.4μm / interference fringe spacing: 7.6 nm) were recorded at a biprism voltage of 35 V using a Gatan CCD camera. The phase shift was extracted from the holograms by using the Fourier transform.

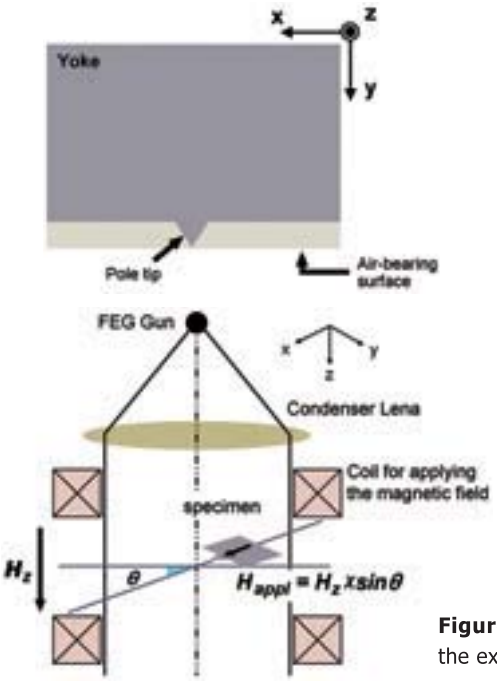

Figure 1. Schematic illustrations of the writer pole and the experimental technique applied in this study.

	Ni-Fe	Co-Ni-Fe
Saturation induction, B_s (T)	2.0	2.3
Coercivity easy axis, H_c-e (A/m)	326	979
Coercivity hard axis, H_c-h (A/m)	80	279
Anisotropy field, H_k (A/m)	279	637
Magnetostriction, λ_s ($\times 10^{-6}$)	15.0	40.0

Table 1. Magnetic properties of the Ni-Fe and Co-Ni-Fe thin films. Their magnetization easy axes correspond to the x axis shown in Fig. 1.

Figures 2(a)–(e) show the reconstructed phase images exhibiting the magnetization process of the Ni-Fe pole tip. In the reconstructed phase image, the direction and density of the contour lines correspond to those of the magnetic flux lines projected along the electron beam [6, 7]. Figure 2(a) shows the reconstructed phase image obtained when the magnetic field is reduced to zero after applying a magnetic field of 24.0 kA/m in the direction indicated in Fig. 2(b), i.e., the remanent state. It is clearly seen that the Ni-Fe pole tip has closure domains, and the magnetic flux rotates in the clockwise direction at the bottom edge of the pole tip. No stray field is observed around the pole tip. The first motions of the domain walls are observed when a magnetic field of approximately 3.2 kA/m is applied, as shown in Fig. 2(b). The area of the domains magnetized in the same direction as that of the applied magnetic field increases, and the center of the closure domains located at the bottom edge of the pole tip is shifted to the left side. These domain wall motions result in the leakage of the stray field from the pole tip. In Fig. 2(c), the rotating feature of magnetic flux disappears at the side edge of the pole tip (indicated by 'I') where the domain walls are pinned. With an increase in the applied magnetic field [Figs. 2(c)–(d)], the domain wall indicated by 'I–II' moves along the side edge of the pole tip so that the stray field becomes stronger. It is found that when a magnetic field is applied, the shape of the domain walls is curved (not straight). This proves that the domain walls are pinned on the side edge of the pole tip. Finally, when the magnetic field is 12.0 kA/m [Fig. 2(e)], the pole tip is fully magnetized in the field of view. Meanwhile, through successive experiments, it was found that the domain structure is reversibly maintained in the remanent state obtained from a different magnetic field history. The magnetization process of the Ni-Fe pole tip was reproducible. Therefore, this result implies that there is no pole erasure in the Ni-Fe pole and agrees with the result obtained from the pole erasure testing.

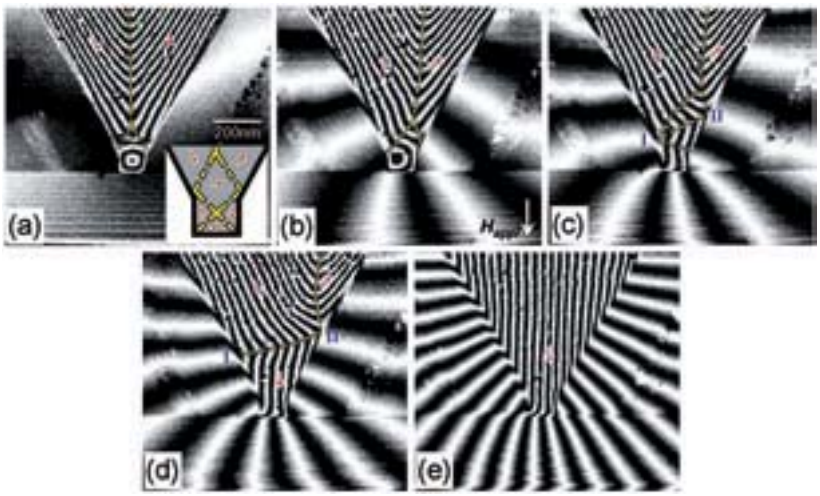

Figure 2. Reconstructed phase images exhibiting the magnetization process of the Ni-Fe pole tip. (a) Remanent state. (b)–(e) are the reconstructed phase images obtained when the applied magnetic field in the specimen plane is 3.2, 4.0, 5.6, and 12.0 kA/m, respectively. The direction of the applied magnetic field is indicated by a white arrow in (b). The domain walls were depicted with the broken yellow lines except the regions where the closure domains exist at the bottom edge of the pole tip in (a) and (b). The direction of magnetic flux lines is indicated by red arrows. Both the domain walls and the direction of magnetic flux lines in the closure domains located at the bottom edge of the pole tip are illustrated in the inset of (a).

Figure 3 shows the reconstructed phase image exhibiting the magnetization process of the Co-Ni-Fe pole tip. The result obtained from the remanent state specimen, whose magnetic field history is the same as that in the case of Fig. 2(a), is shown in Fig. 3(a). The magnetic flux is closed as in the case of the Ni-Fe pole tip, while the domain structure is more complex; rotating features of the magnetic flux are observed (there are two rotation centers). Further, the number of domains magnetized in the direction parallel to that of the magnetization easy axis is large compared to that of the domains of the Ni-Fe pole tip. In the Co-Ni-Fe pole tip, as shown in Fig. 3(b), the first motion of the domain walls is observed when the applied field is approximately 4.0 kA/m, which is a large value compared to that in the case of the Ni-Fe pole tip with a small coercivity. With an increase in the magnetic field applied [Figs. 3(b)–(d)], it is seen that the centers of the closure domains are shifted toward the opposite direction and disappear at the side edges of the pole tip where the domain walls are pinned. The

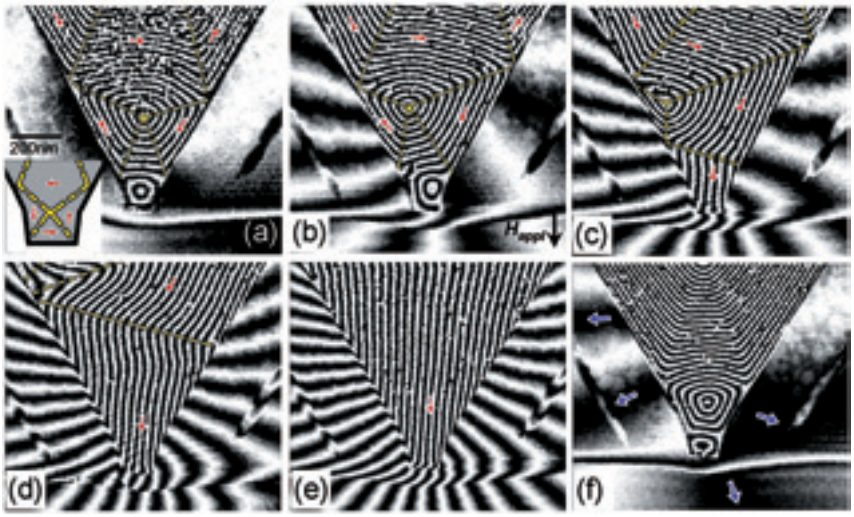

Figure 3. Reconstructed phase images exhibiting the magnetization process of the Co-Ni-Fe pole tip. (a) Remanent state. Inset of (a) shows the positions of the domain walls and the magnetic flux direction at the bottom edge of the pole tip. (b)–(e) are the reconstructed phase images obtained when the applied magnetic field is 4.0, 6.4, 8.0, and 12.0 kA/m, respectively. The direction of the applied magnetic field is indicated by a black arrow in (b). (f) Remanent state obtained after applying a magnetic field of 24.0 kA/m in the direction opposite to that indicated by the black arrow in (b).

magnetic flux lines move in the direction of the applied magnetic field. Finally, the pole tip is fully magnetized as shown in Fig. 3(e). From these results, it is found that the Co-Ni-Fe pole tip is magnetized through a more complicated magnetization process as compared to that of the Ni-Fe pole tip, and the shape of the domain walls observed when the magnetic field is applied is rather straight (not curved). It is considered that these results are attributable to the magnetic properties of the Co-Ni-Fe thin film, i.e., higher coercivity, anisotropy field, and magnetostriction.

Now, we present the result that implies the pole erasure characteristic of the Co-Ni-Fe pole tip. Figure 3 (f) shows the reconstructed phase images obtained from the remanent specimen whose magnetic field history is basically the same as that in the case of Fig. 3 (a), but the direction of the magnetic field initially applied is opposite. It is interesting to note that the Co-Ni-Fe pole tip has different domain structures from those illustrated in Fig. 3(a). The stray field leaking from the pole tip is clearly observed as indicated by blue arrows; this is caused due to the unsymmetrical distribution of the magnetic flux. This result directly indicates that the Co-Ni-Fe pole tip has hysteretic magnetic properties and can cause pole erasure. It is presumably considered that the irreversible and hysteretic feature might be related to the interaction between the domain wall and the side edge of the pole tip as well as the magnetization process of the yoke [8, 9].

To summarize, we have demonstrated that electron holography is very useful in clarifying the domain structure of the nanosized pole tip and its change under the magnetic field by visualizing the detailed magnetic flux distributions. It is expected that the experimental technique presented in this study would provide useful information to clarify and solve problems such as pole erasure in a perpendicular recording system.

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4 Publications

4.1 Journals

Kim, J.J., Hirata, K., Ishida, Y., Shindo, D., Takahashi, M., & Tonomura, A. Magnetic domain observation in writer pole tip for perpendicular recording head by electron holography. *Applied Physics Letters* 92, 162501/1–162501/3 (2008).

Tonomura, A. Development of Electron Holography and Its Applications to Fundamental Problems in Physics. *Japan Society of Applied Physics Inter.* 18, 4-10 (2008).

Tonomura, A. Chen Ning Yang - My Great Adviser. *The Association of Asia Pacific Physical Societies Bulletin* 18, 4, 3-8 (2008).

Tonomura, A. Development of electron phase microscopes. *Nuclear Instruments and Methods in Physics Research A* 601, 203-212 (2009).

Hirata, K., Ishida, Y., Kim, J.J., Kasai, H. Shindo, D., Takahashi, M. & Tonomura, A. Electron holography observation of in-plane domain structure in writer pole for perpendicular recording heads. *J. Appl. Phys* 105, 07D538-531-1507D538-533 (2009).

4.2 Book(s) and other one-time publications

4.3 Oral presentations

Tonomura, A. Quantum World Visualized by Electron Waves The 1st International Symposium on Advanced Microscopy and Theoretical Calculations (AMTC1), Nagoya, Japan, June 29, 2008

Tonomura, A. Electron Phase Microscopy to Observe Superconductivity and Magnetism The 9th International Simposium on Foundations of Quantum Mechanics in the Light of New Technology, Saitama, Japan, August 28, 2008

Tonomura, A. The Quantum World Unveiled by Electron Waves The 9th Asia-Pacific Microscopy Conference(APMC9), Jeju, Korea, November 3, 2008

Ishida, Y., Kim, J. J., Hirata, K., Shindo, D., Takahashi, M., Tonomura, A. Magnetization Process in Writer Pole Tips for Perpendicular Recording Head Studies by Electron Holography The 9th Asia-Pacific Microscopy Conference(APMC9), Jeju, Korea, November 6, 2008

Hirata, K., Kim, J. J., Ishida, Y., Shindo, D., Takahashi, M., Tonomura, A. Electron Holography Observation of In-Plane Domain Structure and Magnetization Process in Writer Pole for Perpendicular Recording Heads 53rd Magnetism and Magnetic Materials Conference (MMM 2008), Austin, USA, November 13, 2008

Tonomura, A. Observation of Vortices Inside Superconducting Thin Films A Scientific Conference in Honor of Alexei Abrikosov's 80th Birthday, Argonne National Laboratory, Illinois, USA, November 7, 2008

Tonomura, A. Electron Phase Microscopy to Observe Superconductivity and Ferromagnetism, AIST-RIKEN Joint WS on "Emergent phenomena of correlated materials", Okinawa, Japan, March 6, 2009

4.4 Posters

Kim, J. J., Hirata, K., Ishida, Y., Shindo, D., Takahashi, M., Tonomura, A. Electron holography of writer pole tip for perpendicular recording head, The Japan Society of Microscopy, the 64th Annual Meeting, Kyoto, May 21–23, 2008

Human Developmental Neurobiology Unit

Principal Investigator:
Dr Gail Tripp

Research Theme:
Reward mechanisms in human behaviour and neuropsychiatric disorders

Abstract

The research of the Human Developmental Neurobiology Unit focuses on the role of reinforcement mechanisms in human behaviour and neuropsychiatric disorders. At present our research efforts are concentrated on attention deficit hyperactivity disorder (ADHD), a prevalent and debilitating disorder first evident in childhood.

Our research hypothesis is that children with ADHD have an altered response to reinforcement which contributes to their symptoms of inattention, overactivity and impulsivity. We will use a range of innovative computer tasks to clarify the nature and extent of this altered reinforcement sensitivity in children with ADHD. Findings from these studies will contribute to increased understanding of the mechanisms underlying altered sensitivity to reinforcement and to the underlying cause(s) of ADHD. We hope this knowledge can then be translated into more effective behavioural and pharmacological interventions for this chronic and debilitating disorder.

1 Staff

Researchers: Dr Emi Furukawa
Dr Keiko Ito
Technical Staff: Ms Yuko Hokama
Mr Naoya Miyagi
Ms Waka Teruya
Research Administrator / Secretary: Ms Mika Matsuda

2 Partner Organizations

University of Otago
Type of partnership: Joint research
Name of principal researcher: Dr Gail Tripp
Name of researchers: Dr Jeff Wickens, Dr Brian Hyland, Dr Brent Alsop, Dr Alison Mercer
Research theme: Cellular and behavioural mechanisms of hyperactivity and movement disorders.

University of State of Rio de Janerio
Type of partnership: Joint research agreement
Name of principal researcher: Dr Gail Tripp
Name of researchers: Dr Egas Caparelli-Daquer, Dr Emi Furukawa
Research theme: Cross-national continuity of altered reward sensitivity in children with ADHD.

3 Activities and Findings

3.1 Preparatory Research Activities

3.1.1. Human Subjects Research Review: Members of the research team have worked closely with the Research Support Team and the OIST Compliance Officer to develop OIST's Human Subjects' Research Regulations and Review procedures in both English and Japanese. Upon finalization of the regulations and procedures Unit staff conducted training workshops for the newly appointed Human Subjects Research Review Committee.

3.1.2 Translation of Research and Assessment Materials: Not all of the experimental and assessment materials required for our research are available in Japanese. Unit members have been actively involved in the translation and back translation of rating scales, test manuals, and experimental tasks. The highly specialized vocabulary of the measures we use, the complexity of the constructs involved, the age of our research participants, together with the need for revision and pilot testing has necessitated this work be carried out internally by our bilingual staff.

3.1.3 Participant Recruitment: As a recently formed team conducting research in an area which, until recently, has received relatively little attention in Japan it has been important for us to make ourselves well known to the local community from which we wish to recruit participants. We have made numerous visits to health, educational, and community organizations to introduce our unit staff and our research goals. This has included a meeting with the Okinawa Board of Education and follow-up visits to the four Education Offices (Kunigami, Nakagami, Naha and Shimajiri). We have attended and presented our proposed research to the Kunigami and Nakagami School Principal meetings. We have also met individually with interested elementary school principals.

3.1.4 Pilot Testing/Focus Groups: Focus groups have been conducted with local parents and teachers to assess their responses to the assessment measures, experimental tasks, and research procedures we utilize in our research. Feedback from these meetings has been incorporated into our research practices. We have begun pilot testing of the assessment procedures and experimental tasks with normally developing children and children with disabilities.

3.1.5 Training Activities/Ongoing Education: Unit members have continued to engage in relevant training activities in preparation for data collection. These activities include both internal workshops together with presentations from relevant local authorities. In addition the PI and post doctoral researchers have attended a number of relevant national and international meetings (e.g., APA Annual Convention, International Neuropsychological Society meeting, European Network for Hyperkinetic Disorders meeting, 17th Convention of the Japanese Society for Child and Adolescent Psychiatry, 17th Convention of Japanese Academy of Leaning Disabilities).

3.2 Outreach Activities

3.2.1 Academic Research Visits: To increase local and national awareness of the Human Developmental Neurobiology Unit and its research goals, unit staff have undertaken academic/research visits to a number of institutions throughout Japan including: University of Ryukyus, Okinawa International University, Tsukuuba University, National Institute of Mental Health, Nara Medical University, Nara Educational University, Kyoto University and Hokkaido University.

3.2.2 Community Outreach Activities: Recruiting children and families to our research will be facilitated by a high level of community awareness regarding our unit activities and confidence in our expertise in the field of ADHD. Activities towards these goals have included community/public talks and the development of parent and teacher resource materials (ongoing) and an accessible unit web site (also ongoing).

3.3 Collaborative Research Activities

3.3.1 Existing Collaborations: In addition to her research activities at OIST the unit PI maintains an active programme of related research at the University of Otago, New Zealand. This research is funded by a three year grant from the Health Research Council of New Zealand. The Co-PI (Dr Alsop) and Research Fellow (Dr Paula Sowerby) make an active contribution to the ongoing research of the

Human Developmental Neurobiology Unit through task development and training assistance.

3.3.2 New Research Collaborations: Through OIST the Human Developmental Neurobiology Unit has established a collaborative research project with the University of State of Rio de Janerio, Brazil. The project "Cross-national continuity of altered reward sensitivity in children with ADHD" brings together researchers from New Zealand, Brazil and Japan. The collaboration was initiated by Dr Emi Furukawa (post doctoral research fellow) who is spending a 4 month period in Brazil establishing the research and investigating additional collaborative research opportunities.

4 Publications

4.1 Journals

Johansen, E.B., Killeen, P.R., Russell, V.A., Tripp, G., Wickens, J.R., Tannock, R., Williams, J., & Sagvolden, T. Origins of altered reinforcement effects in ADHD. *Behav Brain Funct* 5, 7 (2009).

Tripp, G. & Wickens, J.R. Research review: dopamine transfer deficit: a neurobiological theory of altered reinforcement mechanisms in ADHD. *J Child Psychol Psychiatry* 49 (7), 691-704 (2008).

Tripp, G. & Wickens, J.R. Response to William's commentary. *J Child Psychol Psychiatry* 49 (7), 711 (2008).

4.2 Book(s) and other one-time publications

Sowerby, P., & Tripp, G. "Evidence based assessment of ADHD." (pp 209-240) In J. Matson, F. Andrasik, & M. L. Matson. (Eds). Assessing Childhood Psychopathology and Developmental Disabilities. New York: Springer. (2008).

4.3 Oral presentations

Tripp, G. Altered sensitivity to reward: A putative model for attention deficit hyperactivity disorder (ADHD). National Institute of Mental Health, Toyko, Japan, June 27, 2008.

Miyagi, N. ADHD: The disorder and its management. Hopstep NPO, Kodomo no Kuni, Okinawa City, Okinawa, Japan, September 24, 2008.

Tripp, G. Children's Research Centre (Japanese). Okinawa Institute of Science and Technology, Okinawa, Japan, November 9, 2008.

Tripp, G. Dopamine Transfer Deficit: A work in progress. ADHD Research in Japan Meeting. Seaside House, Okinawa, Japan, November 28, 2008.

Tripp, G. Altered reinforcement sensitivity and attention deficit hyperactivity disorder. Hokkaido University, Sapporo, Japan, December 8, 2008.

Tripp, G. In search of the cause of attention deficit hyperactivity disorder (ADHD): Contribution of dopamine and altered reinforcement sensitivity. Towards developing an individual brain... In order to enhance unique characteristics of developmental disorders."「ひとりひとりの脳を育てる… 発達障害のユニークな特性を活かすために」プログラム. Hokkaido University, Sapporo, Japan December 9, 2008.

Tripp, G. Contribution of neuroscience to understanding human behaviour. United Nations Global Seminar Series. Seaside House, Okinawa, Japan, December 19, 2008.

Tripp, G. Neuropsychological research on ADHD. Meiji-Yasuda Health Foundation, Ginowan. Okinawa, Japan, February 12, 2009.

Furukawa, E., Robinson, T., Tripp, G. A longitudinal analysis of cognitive performance in ADHD: Group vs individual effects. International Neuropsychological Society, Atlanta, Georgia, USA, February 13, 2009.

Tripp, G. The importance of English in a global society. 2nd Forum on English Language Education in Okinawa. Okinawa City, Okinawa, Japan, March 18, 2009.

Furukawa, E. Altered reinforcement mechanisms in ADHD: Cross national continuity, interaction with environment, emotional and behavioral correlates. Neuroscience Center, LABS-D'Or Hospital Network, Rio de Janeiro, Brazil, March 20, 2009.

4.4 Posters

Nothing to report.

5 Intellectual Property Rights and Other Specific Achievements

Nothing to report.

6 Meetings and Events

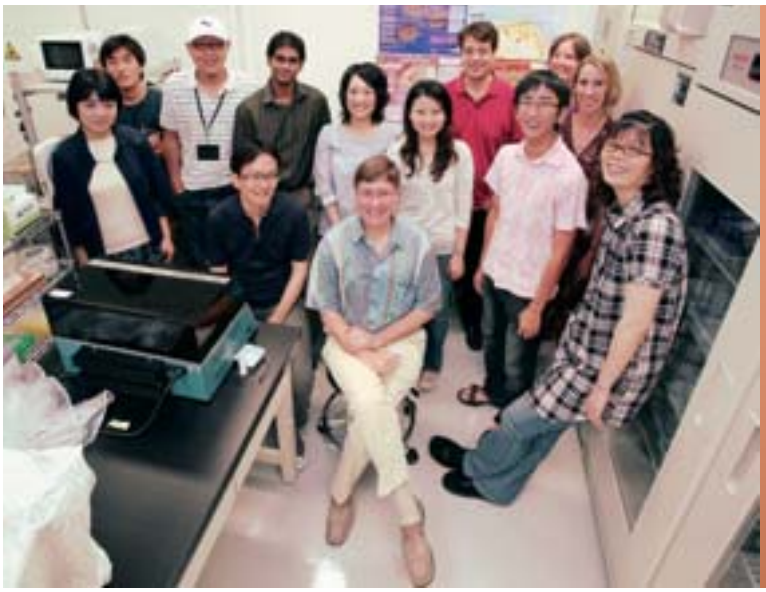
6.1 ADHD Research in Japan

Date: November 28th 2008
Venue: OIST Seaside House
Speakers: Yuki Inoue, MD,
National Institute of Mental Health, National Center of Neurology and Psychiatry (NCNP)
Makiko Kaga, MD, PhD,
National Institute of Mental Health, National Center of Neurology and Psychiatry (NCNP)
Taishi Masunami, PhD, University of Tsukuba
Shintaro Funahashi, PhD, Kyoto University
Hidemi Iwasaka, MD, PhD, Nara University of Education
Hideki Negoro, MD, PhD, Nara Medical University
Junzo Ilda, MD, PhD, Nara Medical University
Gail Tripp, PhD, Okinawa Institute of Science and Technology
Other remarks: Research meeting
Dr Keiko Ito (post doctoral research fellow) took a lead role in the organization (academic) of this meeting.

7 Special Acknowledgment

We would like to take this opportunity to thank all the individuals and organizations who have assisted us in establishing our research programme here in Okinawa. We would especially like to acknowledge the help we have received from Miyazato Elementary School.

Neurobiology Research Unit



Principal Investigator:
Jeff Wickens

Research Theme:
Cellular mechanisms of learning and adaptive behaviour

Abstract

The overall aim of the Neurobiology Research Unit is to elucidate the cellular mechanisms of learning and adaptive behaviour in the brain. We focus on the mammalian basal ganglia, a set of brain structures implicated in reward and intentional action. Previously we found that reward causes physical changes in the neural connections of the basal ganglia, which are associated with learning. Now we aim to discover the mechanisms that govern these changes in neural connections, concentrating on the actions of neurochemicals - such as dopamine - that are released by rewards. Our hypothesis is that molecular signaling networks activated by dopamine define precise rules for activity-dependent synaptic plasticity. We are investigating synaptic plasticity using electrophysiological, molecular and computational approaches at multiple levels of analysis. We are undertaking studies using 2-photon microscopy to measure events taking place at individual dendritic spines. Conventional patch-clamp recording is being used to measure synaptic plasticity. Lines of transgenic mice have been established in which different types of cells can be definitively identified. Using these approaches we are investigating (i) cell-type specific differences in synaptic plasticity, (ii) molecular mechanisms involved in induction of synaptic plasticity, and (iii) precise timing requirements for synaptic plasticity. The long term aims of these experiments are to determine rules for induction of synaptic plasticity and to elucidate their underlying cellular mechanisms. We have also undertaken computational and theoretical studies of basal ganglia function, and initiated system-level studies of dopamine release in during behaviour. The research has the forward aim of understanding disorders of the dopamine system - such as Parkinson's disease and attention-deficit hyperactivity disorder - and finding better treatments for such disorders.

1 Staff

Researchers: Dr Tomomi Shindou
Dr Catherine Vickers
Dr Mayumi Ochi-Shindou
Dr Karen Rommelfanger (departed 31 January 2009)
Dr Adam Ponzi

Technical staff: Mr Kiyoshi Baba
Ms Michelle Callahan
Dr Saori Miura
Mr Prageeth Saraka Wimalaweera

Research assistants / graduate students: Mr Takashi Nakano (based in Doya Unit)
Mr Yu-Ting Li
Ms Yi-Ling Huang

Research administrator / secretary: Ms Yukako Suzuki

2 Partner Organizations

University of Otago, New Zealand
Type of partnership: Joint Research
Name of principal researcher: Jeff Wickens
Name of researchers: Dr Brian Hyland, Dr Brent Alsop, Dr Alison Mercer, Dr Gail Tripp
Research theme: Cellular and Behavioural Mechanisms of Hyperactivity and Movement Disorders

Institute of Biomedical Engineering, National Cheng Kung University, Taiwan
Type of partnership: Research Collaboration (currently being set up)
Name of principal researcher: Dr Jason Chen
Name of researcher: Mr Yu-Ting Li
Research theme: Research on neuroplasticity using wireless dopamine sensing and microPET

3 Activities and Findings

The Neurobiology Research Unit was established January 29, 2007. During the first year of operation, the laboratory interior was completed and the equipment was commissioned. Staff were recruited and the Unit grew rapidly to its current size. Initial research activity has focussed on experimental studies of synaptic plasticity in the corticostriatal pathway, and theoretical studies of striatal network dynamics and reinforcement mechanisms important in learning. Activity has been conducted in the following main areas:

3.1 Measurement of timing requirements for induction of synaptic plasticity

Synaptic plasticity mechanisms are thought to operate at the level of individual synapses. We hypothesize that the selection of particular synapses for modification during learning involves activity-dependent plasticity with precise timing requirements. Among other factors, the timing of presynaptic and postsynaptic activity is likely to be crucial. We have studied dendritic mechanisms associated with spike-timing dependent plasticity (STDP) in this pathway. Using whole-cell recording from striatal projection neurons in adult mice we measured STDP over a range of different timing and excitation conditions. We found that long-term depression (LTD) of corticostriatal inputs was maximally induced by pairing protocols in which the cortically evoked EPSP preceded postsynaptic action potentials by 10 ms (pre-post protocol). Post-pre protocols, or stimulation of the pre- or post-synaptic sites alone, induced no change in the cortical EPSP. The LTD was blocked by the intracellular calcium chelators, BAPTA or EGTA, suggesting a requirement for elevated calcium for induction of LTD.

3.2 Measurement of cell-type specific differences in synaptic plasticity

We have initiated studies of synaptic plasticity in striatal cells using electrical field stimulation and electrophysiological recording in brain slices from transgenic mice that express fluorescent markers in specific cell types. Transgenic mouse lines have been established, which selectively express green fluorescent protein (GFP) in subtypes of striatal spiny cells, so that we are now able to definitively identify the neurons from which records have been obtained. In the first phase of this research we developed plasticity-inducing stimulation protocols for mouse brain slices. We have tested the hypothesis that dopamine differentially regulates synaptic plasticity in dopamine D1a versus dopamine D2 receptor expressing subtypes of striatal neuron, by comparing the effects of the same induction protocols on such neurons identified by intracellular labeling and by expression of GFP. We found that STDP protocols produced LTD in dopamine D-1a receptor-expressing neurons but not in dopamine D-2 cells.

Induction of plasticty using high frequency cortical stimulation (100 Hz) produced a different pattern of results in which potentiation predominates and D1a and D2 cells display cell specific responses. D2 cells potentiate to a significantly higher degree that the D1a cells, suggesting that afferent information integration and processing is different in the two cell types. Interestingly, the potentiation observed in the D2 expressing cells was not sensitive to the specific D2 receptor antagonist sulpiride (10μM). However, application of the A_{2A} specific antagonist ZM241385 (1μM) resulted in a significant reduction in the degree of potentiation seen.

Our ability to definitively identify morphologically similar cells by their expression of GFP is a powerful new technique that is adding important new understanding. Our data suggest that there are specific differences in synaptic integration between D1a and D2 cells and further work will be undertaken to investigate these differences.

3.3 Investigation of dendritic mechanisms underlying synaptic plasticity

The molecular mechanisms underlying plasticity operate at the level of individual dendritic spines, which receive synaptic inputs from glutamatergic and dopaminergic synapses. As noted above, intracellular Ca^{2+} levels play a key role in the induction of synaptic plasticity in the corticostriatal pathway, and may be the basis of the temporal requirements observed. To investigate the mechanism underlying the spike-timing dependence of LTD, we have initiated studies using 2-photon microscopy to measure intracellular Ca^{2+} concentration in individual dendritic spines. Dendritic spine Ca^{2+} signals associated with the LTD-induction protocols were measured. Pre-post protocols that induced LTD were associated with larger spine Ca^{2+} transients than post-pre protocols that produced no LTD. These findings suggest a Ca^{2+} threshold for induction of LTD in the corticostriatal pathway.

3.4 Theoretical and computational studies of the basal ganglia

Our electrophysiological experiments are being conducted in parallel with computational modeling of the network of phosphatases and kinases present in the dendritic spines of the spiny neurons of the striatum (in collaboration with the Doya Unit). In addition we have been developing computer simulations of network activity in striatal inhibitory networks. We found that inhibitory networks of deterministically interacting spiking neurons show highly irregular firing composed of dynamically switching coherently bursting cell assemblies if the network has sparse to intermediate connectivity and the levels of cortical excitation and network inhibition are balanced so that the cells are near the threshold between quiescent and firing states. Numerical simulation results are in good qualitative agreement with experimental studies and support a new view of the dynamical behaviour of the striatum underlying its information processing operations.

At a more general level, we have contributed to the development of a neurobiological hypothesis of altered reinforcement mechanisms in attention deficit hyperactivity disorder (in collaboration with the Tripp Unit). We proposed that a number of symptoms of the disorder could be interpretted as due to a failure of dopaminergic responses to established reinforcers to transfer to new predictors of reinforcement. This model makes a number of specific behavioural and neural predictions that are testable by experiment.

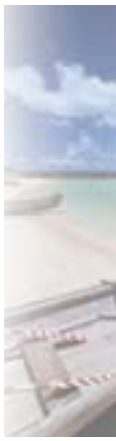
3.5 New areas of research activity

We have recently initiated two new areas of study that will complement our ongoing research program. We are developing our ability to use fast-scan cyclic voltammetry (FSCV) to measure dopamine release in awake-behaving rats using carbon fibre microelectrodes. We have previously shown that dopamine acts at the cellular level to strengthen synapses in the striatum of the forebrain. These actions of dopamine on synaptic strength provide a cellular mechanism for reward-related learning. In this mechanism, precisely timed and pulsatile increase in dopamine concentration are critical for correct processing reward. This technique will enable us to investigate the timing of the dopamine signal at the systems level. We have developed hardware and software to conduct FSCV and perform principal components regression to better identify the chemicals detected. We are also developing our ability to express in selected neurons the algal protein Channelrhodopsin-2, a rapidly gated light-sensitive cation channel, by using lentiviral gene delivery. We have so far expressed the protein in cultured striatal and cortical neurons and demonstrated light-induced activation. This technique will enable temporally precise, non-invasive control of activity in well-defined neuronal populations.

4 Publications

4.1 Journals

Joshua, M., Adler, A., Prut, Y., Vaadia, E., Wickens, J.R., & Bergman, H. Synchronization of midbrain dopaminergic neurons is enhanced by rewarding events. *Neuron* In Press (2009).



Pan, W.X., Schmidt, R., Wickens, J.R., & Hyland, B.I. Tripartite mechanism of extinction suggested by dopamine neuron activity and temporal difference model. *J Neurosci* 28 (39), 9619-9631 (2008).

Ponzi, A., & Wickens, J.R. Cell assemblies in large sparse biologically realistic networks of spikining neurons. *Advances in Neural Information Processing* In Press (2009).

Schulz, J.M., Redgrave, P., Mehring, C., Aertsen, A., Clements, K.M., Wickens, J.R., & Reynolds, J.N.J. Short latency activation of striatal spiny neurons via subcortical visual pathways. *J Neurosci.* In Press (2009).

Shindou, T., Arbuthnott, G.W., & Wickens, J.R. Actions of Adenosine A2A Receptors on Synaptic Connections of Spiny Projection Neurons in the Neostriatal Inhibitory Network. *J Neurophysiol* 99 (4), 1884-1889 (2008).

Tripp, G., & Wickens, J.R. Research review: dopamine transfer deficit: a neurobiological theory of altered reinforcement mechanisms in ADHD. *J Child Psychol Psychiatry* 49 (7), 691-704 (2008).

Wickens, J.R. Toward an anatomy of disappointment: reward-related signals from the globus pallidus. *Neuron* 60 (4), 530-531 (2008).

Wickens, J.R. Synaptic plasticity in the basal ganglia. *Behav Brain Res* 199 (1), 119-128 (2009).

4.2 Book(s) and other one-time publications

Shindou, T., Arbuthnott, G.W., Wickens, J.R. Neuromodulation and neurodynamics of striatal inhibitory networks: implications for Parkinson's disease. In Cortico-Subcortical Dynamics in Parkinson's Disease. Editor: Tseng, Kuei-Yuan., *Humana Press & Springer Editorials* Chapter 14 / ISBN: 978-1-60327-251-3 2009

Shindou, T., Wickens, J.R. Drugs for Motor disorders. In Encyclopedia of neuroscience, Vol. 4, Binder, Marc D.; Hirokawa, Nobutaka; Windhorst, Uwe (Eds), *Springer*, DOI 10.1007/ ISBN: 978-3-540-29678-2_1625 2009

4.3 Oral presentations

Shindou, T. Patch-clamp recording with two-photon microscopy, Ryukyu University, Faculty of Science, Okinawa, Japan., January 25, 2008.

Wickens, J.R. Synaptic plasticity and behaviour, Okinawa Computational Neuroscience Course 2008, OIST Seaside House, Okinawa, Japan., June 17, 2008.

Wickens, J.R. Neurobiology Research at OIST, The Asia Youth Exchange Program, OIST Uruma City Laboratory, Okinawa, Japan., August 18, 2008.

Wickens, J.R. Time, space and dopamine, International Symposium on Drug Addiction: Mechanisms and Therapeutic Approaches. The Chinese Academy of Sciences, Kunming, Yunnan, P.R. China. October 10-13, 2008.

Wickens, J.R. How the brain learns at the level of nerve cells, AGENA Junior High School, Agena Uruma City, Okinawa, Japan., October 30, 2008.

Ponzi, A. The formation of cell assemblies in biologically realistic networks of inhibitory neurons OIST Retreat, OIST Seaside House, Onna-Village, Okinawa, Japan., October 30-31, 2008.

Wickens, J.R. Dynamics of lateral inhibition in the neostriatum: winner-takes-all, winners-share-all, or winnerless competition?, Center for Molecular and Behavioral Neuroscience, Rutgers, The State University of New Jersey, Newark, NJ U.S.A., November 12, 2008.

Wickens, J.R. The basal ganglia in habit formation, The Basal Ganglia in Movement, Behaviour and Emotions, CIREN, La Habana, CUBA., December 1, 2008.

Ponzi, A. Attractor switching in inhibitory networks of spiking neurons, Research Institute for Electronic Science, Hokkaido University, Sapporo City, Japan., January 16, 2009.

4.4 Posters

Shindou, T., Ochi-Shindou, M., Wickens, J.R. The dopaminergic contribution to spike-timing dependent plasticity in the corticostriatal pathway, Japan Neuroscience Society, Tokyo, Japan., July 9, 2008 in English.

Vickers, C., Nakano, T., Wickens, J.R. The roles of dopamine D1 and D2 receptors in synaptic plasticity in the corticostriatal system, Forum of European Neuroscience, Geneva, Switzerland., July 12, 2008 in English.

Ponzi, A., Wickens, J.R. Multiscale switching cell assembly clusters naturally emerge in simulations of random inhibitory networks of spiking neurons, The Summer Workshop on Mechanisms Brain and Mind, Sapporo, Hokkaido, Japan., August 9, 2008 in English.

Shindou, T., Ochi-Shindou, M., Wickens, J.R. Two-photon imaging of Ca^{2+} dynamics in dendrites of medium spiny neurons associated with spike-timing dependent synaptic plasticity in the mouse neostriatum, World Molecular Imaging Congress, Nice, France., September 10, 2008 in English.

Ochi-Shindou, M., Shindou, T., Wickens, J.R. Dopamine modulates spike-timing-dependent synaptic plasticity in the neostriatum of adult mice, Society for Neuroscience, Washington DC, USA. November 15, 2008 in English.

Shindou, T., Ochi-Shindou, M., Wickens, J.R. Two-photon imaging of calcium dynamics in dendrites of medium spiny neurons associated with spike-timing dependent synaptic plasticity in the mouse neostriatum, Society for Neuroscience, Washington DC, USA., November 15, 2008 in English.

Vickers, C., Nakano, T., Wickens, J.R. Dopamine dependent plasticity in the corticostriatal system, Society for Neuroscience, Washington DC, USA., November 15, 2008 in English.

Ponzi, A., Wickens, J.R. Multi-scale switching cell assembly clusters naturally emerge in simulations of random inhibitory networks of hodgkin huxley neurons, Society for Neuroscience, Washington DC, USA., November 18, 2008 in English.

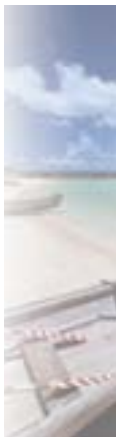
Ponzi, A., Wickens, J.R. Cell assemblies in large sparse biologically realistic networks of spiking neurons, Neural Information Processing Systems, Vancouver, Canada., December 8, 2008 in English.

Ponzi, A., Wickens, J.R. Complex multi-scale identity-temporal patterns and assemblies in inhibitory networks of spiking neurons, The Winter Workshop on Mechanisms Brain and Mind, Rusutsu, Hokkaido, Japan., January 9, 2009 in English.

Ponzi, A., Wickens, J.R. Cell assemblies and complex identity-temporal patterns in inhibitory networks of spiking neurons, The Systems Neurobiology Spring School, Kyoto, Japan., March 13-14, 2009 in English.

5 Intellectual Property Rights and Other Specific Achievements

Nothing to report.



6 Meetings and Events

6.1 OIST Seminar

Date: April 18, 2008
Venue: Uruma City Laboratory, OISTPC
Speaker: Dr Schuichi Koizumi, Professor of the Department of Pharmacology,
Faculty of Medicine at University of Yamanashi (Japan).

Date: October 8, 2008
Venue: Uruma City Laboratory, OISTPC
Speaker: Dr Mark Wightman,
Professor of the Department of Chemistry at University of North Carolina Chapel Hill (U.S.A.).

Date: February 13, 2009
Venue: Uruma City Laboratory, OISTPC
Speaker: Dr Tomomi Shindou,
Okinawa Institute of Science and Technology, Neurobiology Research Unit.

6.2 Okinawa Computational Neuroscience Course 2008

Date: June 16 – July 3, 2008
Venue: Seaside House, OISTPC
Co-organizers: Dr Erik De Schutter, Dr Kenji Doya, Dr Klaus Stiefel,
Okinawa Institute of Science and Technology
Co-sponsors: Nara Institute of Science and Technology
Japanese Neural Network Society
Speaker: Invited faculty

6.3 The Demonstration Experiments of DNA for junior high school students

Date: February 5–6, 2009
Venue: KUBURA Junior High school, YONAGUNI Junior High School, YONAGUNI Island, Okinawa.
Co-organizer: Dr Mary-Ann Price, Okinawa Institute of Science and Technology,
Developmental Signaling Unit.
Co-sponsor: "The Okinawa Prefectural Assembly" that promote the establishment of
the graduate university in Okinawa.
Speaker: Dr Catherine Vickers,
Okinawa Institute of Science and Technology, Neurobiology Research Unit.

G0 Cell Unit

Principal Investigator:
Mitsuhiro Yanagida

Research Theme:
Cellular Strategy for Maintaining Starved G0 Arrest and Promoting Vegetative Proliferation

Abstract

Our research goal is to understand the molecular mechanisms of regulations in cellular quiescence and proliferation, and also in maintenance of the quiescent cells. The quiescent state, the so-called G0 phase, is a central problem in biology as well as cell growth and multiplication. It is not well known how cells are able to control and execute an arrest at the G0 state, and how cell growth and division resume when the nutritional environment improves. As a model system, we have adopted a simplest eukaryote, fission yeast *Schizosaccharomyces pombe*.

This year we further identified and analyzed genes that are involved in the entry to, maintenance of, and exit from the G0 state. From 610 strains of temperature-sensitive mutants, we have so far found 7 genes required for entry to the G0 state and 26 genes required for the maintenance of G0 cells. We found that the stress-responding MAP kinase pathway, actin-regulated endocytosis and vacuole fusion regulation are involved in the entry to quiescence. We also found that the regulation of RNA polymerase II by phosphorylation/dephosphorylation plays a major role in the switch between proliferation and quiescence.

We have also proceeded in the analyses of previously identified important genes for the G0 phase. We isolated the rapamycin sensitive mutants of the components of the TOR complex, a key factor for signal transduction as the nutrient-response. We identified putative transcriptional targets of Klf1, a transcriptional regulator that is required for the viability in the G0 phase. From a ubiquitin-dependent proteasome mutant, we found that the autophagy system selectively degraded mitochondria to reduce oxidative stress to maintain the G0 cells. Finally, we identified a mutant that is resistant to an immunosuppressant, tamoxifen, which causes lethal damage to G0 cells.

Furthermore, we have made substantial progress in metabolomic analysis utilizing mass spectrometry. We have also developed useful software to analyze the data. This analysis has been applied to several mutants we have identified as defective in G0 maintenance.

1 Staff

Okinawa

Group leader: Mizuki Shimanuki
Researchers: Koji Nagao
Kojiro Takeda
Technical staff: Sakura Kikuchi
Ayaka Mori
Aya Kokubu
Risa Uehara
Tomas Pluskal
Bryan Mathis
Alejandro Villar Briones
Research assistant / graduate student: Kenichi Sajiki

Research administrator / secretary: Tomomi Teruya

Kyoto
Researchers: Takeshi Hayashi
Kumiko Ohta (from March, 2009)
Research administrator / secretary: Yukari Matsushita

2 Partner Organizations

**Kansai Advanced Research Center,
National Institute of Information and Communications Technology**
Type of partnership: Collaboration
Name of principal researcher: Yasushi Hiraoka
Name of researcher: Yuji Chikashige
Research theme: cDNA microarray analysis of *S. pombe* during re-entry into proliferation from G0-like stage

Department of Biophysics and Biochemistry, Graduate School of Science, University of Tokyo
Type of partnership: Technical Help
Name of principal researcher: Masayuki Yamamoto
Name of researcher: Kayoko Tanaka (Present: Department of Biochemistry, Univesity of Leicester)
Research theme: Gene disruption analysis of *S. pombe*

Chemical Genetics Laboratory, Discovery Research Institute, Wako Institute, Riken
Type of partnership: Collaboration
Name of principal researcher: Minoru Yoshida
Name of researcher: Shinichi Nishimura
Research theme: Search for chemicals and drugs that are inhibitory to the maintenance of G0 state

Division of Molecular Life Science, Bioinformation and Molecular Science Course, Graduate School of Life Science, Hokkaido University
Type of partnership: Collaboration
Name of principal researcher: Chikashi Obuse
Research theme: Proteomic analysis of *S. pombe* G0 cells

Laboratory of Molecular Inheritance, Graduate School of Biostudies, Kyoto University
Type of partnership: Collaboration
Name of researcher: Takahiro Nakamura
Research theme: Metabolomic analysis of *S. pombe* cells using LC-MS mass spectrometer

Laboratory of Molecular Inheritance, Graduate School of Biostudies, Kyoto University
Type of partnership: Collaboration
Name of researcher: Yohta Fujita
Research theme: Identification of novel kinetochore components using LC-MS mass spectrometer

Bioneer Corporation (BIONEER) and Korea Research Institute of Bioscience and Biotechnology (KRIBB)
Type of partnership: Collaboration
Name of researchers: K.-L. Hoe, D.U. Kim, and H. Park
Research theme: Analysis of systematic genome wide haploid deletion mutants in Schizosaccharomyces pombe for drug sensitivity of G0 cells

The Department of Gene Mechanisms, Graduate School of Biostudies, Kyoto University
Type of partnership: Collaboration
Name of researcher: Junko Kanoh
Research theme: Analysis of TOR complexes by specific and common subunits

Laboratory of Molecular Inheritance, Graduate School of Biostudies, Kyoto University
Type of partnership: Collaboration
Name of researcher: Norihiko Nakazawa
Research theme: Identification of condensin accumulation components using LC-MS mass spectrometer

3 Activities and Findings

3.1 Use of a temparature-sensitive mutant collection to screen the genes required for entry into and/or maintenance of quiescence under nitrogen starvation.

Transition from proliferation to quiescence brings about extensive changes in cellular behavior and structure. However, little is known about the genes crucial for quiescence. To identify such genes, we characterized 610 temperature-sensitive (ts) mutants (Sajiki et al. 2009). Twelve of the 610 ts mutants have significantly decreased cell viability (<35%) under nitrogen starvation without temperature shift from the permissive condition (26°C) for the vegetative proliferation. We established that eight strains of those 12 corresponded to seven distinct mutant genes. Those seven genes required for "entry into quiescence" could be classified into three groups on the basis of known functions of their products. The first group is stress-responding MAP kinase pathway genes, *sty1* and *wis1*, encoding MAPK and MAPKK, respectively. Those mutants were defective in arresting cell growth (**Figure 1A**), down-regulating a mitotic cyclin Cdc13 and up-regulating a CDK inhibitor Rum1, as responses to nitrogen starvation. The second group consists of genes involved in vesicle fusion. *vam6* and *vps11* encode a guanyl-nucleotide exchange factor and a RING finger protein, respectively, and both are subunits of the HOPS (homotypic fusion and vacuole protein sorting) complex. *ypt5* encodes a Rab-related GTPase. Under nitrogen starvation, *vam6* and *vps11* mutant cells could not perform pre-quiescence division and numerous small vesicles were accumulated in the cytoplasm (**Figure 1B**). *ypt5* mutant cells could divide once upon nitrogen starvation, but they could not form the increased numbers of normal vacuoles that are observed in the wild type quiescent cells. The third group genes, *end4* and *wsp1*, encode a homolog of Huntingtin-interacting protein and a homolog of WASP (Wiskott-Aldrich syndrome) protein, respectively, both of which are involved in actin-interacting endosome function. These mutant cells under nitrogen starvation were osmo-sensitive (**Figure 1C, D**).

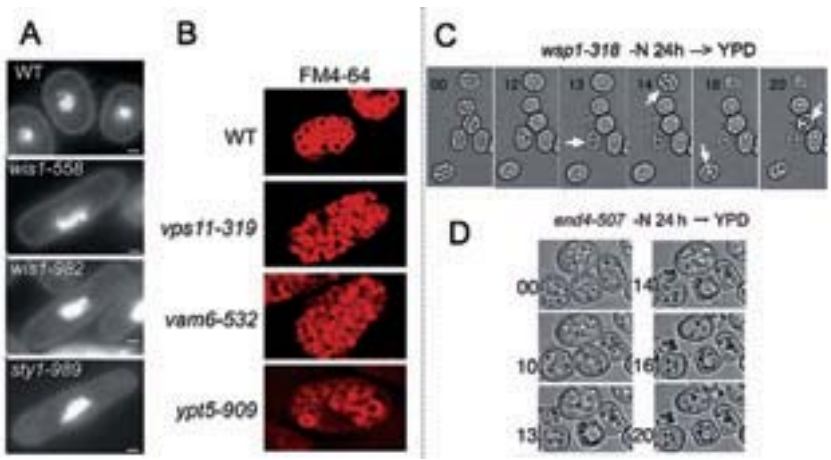


Figure 1. Phenotype of the mutants that have defects in the entry to quiescence. A: MAP kinase mutants cannot stop cell growth under nitrogen starvation. B: Vacuole fusion mutants. C, D: endosome mutants showed osmo-sensitivity.

In total, 610 strains were incubated at 37°C for 3 days in the –N (nitrogen deficient) medium after culture for 1-day quiescence entry at 26°C. Of these, 164 strains showed significantly decreased viability (<50%) in this second-stage of culture for "quiescence-maintenance". An unexpected conclusion was that approximately three-quarters of those ts genes required for proliferation might be dispensable for maintaining quiescence. We attempted to identify the mutant genes for 34 strains by gene cloning, tetrad dissection and nucleotide sequence determination of the mutant genes. Twenty-six genes were identified and those functions covered a broad range of cellular activities involving the cell surface, cytosol, cytoplasmic organelles and nucleus. These gene products and their functions are summarized in **Figure 2**.

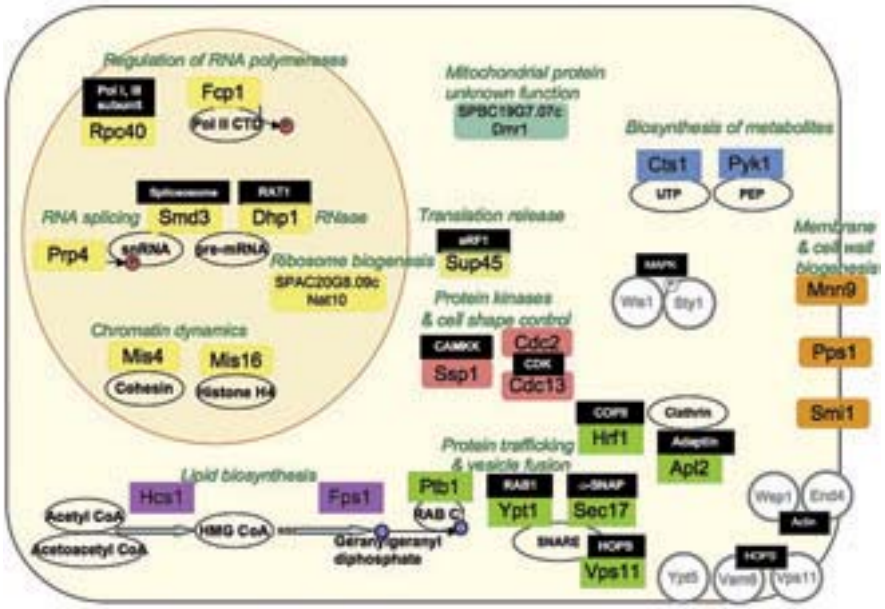


Figure 2. Identified genes involved in the regulation of quiescence. Twenty-six genes required for the maintenance of *S. pombe* G0. Seven genes for the quiescence entry are also shown (gray circles).

fcp1 is one of the most interesting genes among them, as it encodes a protein phosphatase that regulate the mRNA transcription activity of RNA polymerase II by dephosphorylation of the C-terminal repeat domain. Contrary to the prediction of Fcp1 acting on general transcriptional machinery, the effects on the transcriptome of the *fcp1-452* ts mutant were surprisingly selective (**Figure 3A**). Moreover, Fcp1 differentially affects transcription of genes involved in quiescence and proliferation (**Figure 3B, C**). Our results indicate that the balance between proliferation and quiescence is controlled by RNA polymerase II through the regulation of the state of its CTD phosphorylation by Fcp1 phosphatase. Fcp1 plays a major role in transcriptional support for the quiescent state because its mutant lacks the principal intracellular features of quiescence (**Figure 3D, E**).

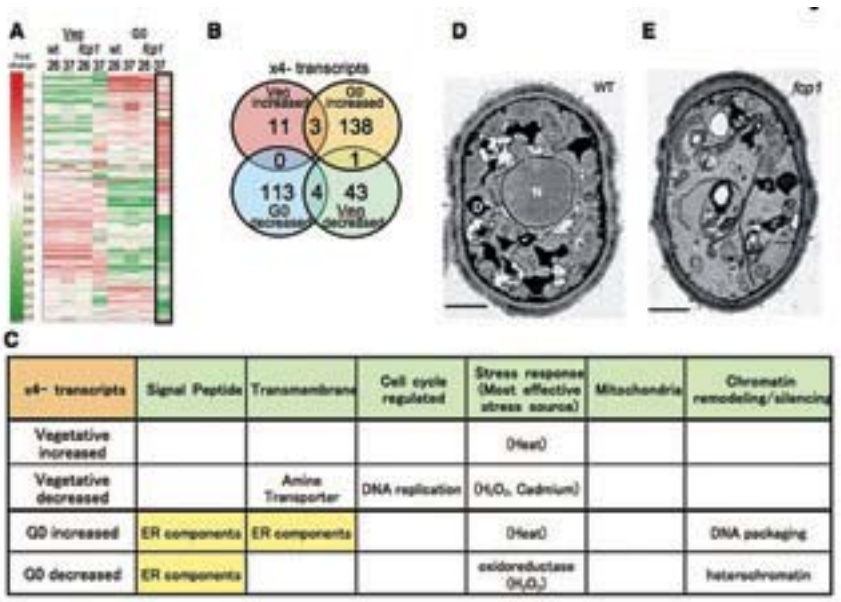


Figure 3. Fcp1 CTD phosphatase affects a broad range of transcripts in vegetative and G0. (A) Transcriptomic patterns of WT and *fcp1* ts mutant in vegetative or G0 at 26 and 37°C. Color panel indicates relative increase (red) and decrease (green) to the median (white) of eight different culture conditions for each transcript. Transcripts of vegetative cultures were obtained by shifting from 26°C to 37°C (26°C in control) for 4 h in the vegetative medium. Transcripts of G0 cultures were obtained by shifting from 26°C (pre-culture for 24 h in the –N medium) to 37°C (26°C in control) for 24 h in the G0 medium. (B) Classification of increased or decreased transcripts in *fcp1* mutant at 37°C in the vegetative or the G0 medium. (C) The ontology of significantly affected genes for each category under each condition. Note that significant number of ER component genes was affected by *fcp1* mutation at

37°C in G0 medium. (D, E) Thin section electron microscopy of WT (D) and *fcp1* mutant (E) cells at 37ºC in the G0 medium. Note in the *fcp1* mutant cell, ER like membrane structure is abnormally developed.

It is surprising that a single *fcp1* mutation causes very large changes in the levels of mostly non-overlapping transcripts in both proliferating and quiescence cells at the restrictive temperature (**Figure 3B**). It remains to be investigated how specific transcripts implicated in DNA replication, membrane transport, trafficking, stress responses, mitochondrial structure and function, and chromatin remodeling are regulated differentially in proliferation and quiescence. We published these results in JCS.

Although we already have identified some important genes for G0 from these analyses, many of the ts mutants still remain to be analyzed. We have recently employed a whole genome sequencing method enabled by next generation DNA sequencer, to facilitate the detection of mutation sites and identification of the responsible mutant genes for the ts phenotype.

3.2 Isolation of rapamycin-sensitive mutants of TOR complex subunits, key factors of signal transduction in response to the nutritional environment.

We found that a ts mutant of the *tor2* gene, encoding a phosphatidyl inositol kinase-related kinase (PIKK), plays an important role in the nutrient signaling pathway. This ts mutant, *tor2-287*, failed to recover from G0-like arrest at 36°C in rich medium, consistent with the notion that the mutant cells fail to recognize the presence of a nitrogen source at the restrictive temperature. The mutant was hypersensitive to rapamycin, an immunosuppressant utilized for cancer therapy, and revealed a G0-like phenotype in the presence of rapamycin at a permissive temperature, 26°C. This mutation is unique, as previously isolated *tor2* mutants are insensitive to rapamycin. Furthermore, we determined the molecular organization of TOR complexes (TORCs) in *S. pombe* (**Figure 4A;** Hayashi et al., 2007), by mass spectrometry-based identification of the proteins in the co-immuno precipitants.

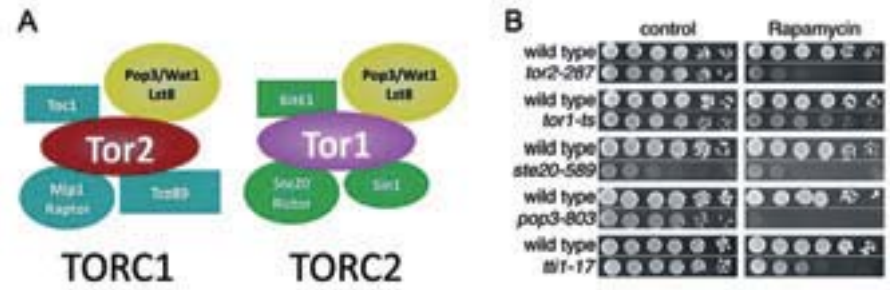
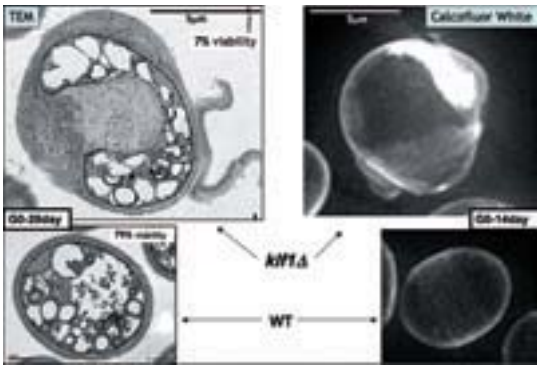


Figure4. Organization of TOR complexes in *S. pombe* and rapamaycin sensitivity of TORC mutants. A: Two TOR complexes. B: Rapamycin sensitivity of the isolated mutants.

We isolated TORC mutants from our ts mutant library (*ste20* and *pop3*) or by site-directed mutagenesis (*tor1*). Tor1 and Ste20 are TORC2-specific subunits, and Pop3 is a common subunit of TORC1 and TORC2. We found that these mutants were hypersensitive to rapamycin (**Figure 4B**). In addition, we isolated a *tti1* mutant by PCR-based random mutagenesis. Tti1 is a subunit of the Tel2 complex, which is a common partner of PIKKs (Tor1, Tor2, Rad3/ATR, Tel1/ATM, Tra1 and Tra2). This *tti1* mutant was also sensitive to rapamycin, indicating that Tti1 is functionally related to TORCs. Analyses of these TORC-related mutants (including *tor2* mutant) will provide a clue to the role of TORCs in proliferating and G0-arrested cells.

3.3 Analysis of Klf1, a putative transcriptional regulator that is required for the normal establishment and/or maintenance of the quiescent cell state under nitrogen starvation

We previously identified 12 genes that are not required for proliferation but are required for maintaining cell viability under nitrogen starvation through gene deletion experiments (Shimanuki et al. 2008). Those genes encode putative transcriptional regulators, phosphorylation/signal transduction regulators and functionally unknown but evolutionarily conserved proteins. First, we further analyzed Klf1, a putative transcriptional regulator with C2H2-type zinc finger motifs that have similarity with those in the krüppel-related family of proteins. Transcription of the *klf1* gene itself was approximately 10 fold induced by nitrogen starvation. The gene deletion mutant of *klf1* arrested its cell cycle under nitrogen starvation but gradually deposited unidentified materials outside of the plasma membrane,



and lost cell viability significantly earlier than the wild type strain. Overproduction of cell wall materials like chitin is suggested by the signal stained with calcofluor white fluorescent dye (**Figure 5**) and also by the accumulation of N-acetyl-glucosaminate detected from the mass spectrometry analysis of the metabolome. S-adenosyl methionine was also accumulated in the *klf1*Δ mutant.

Figure 5. Abnormal cell wall-like materials were deposited in the *klf1-delta* mutant cells

We identified 14 and 11 genes whose transcript level were increased or decreased, respectively, by the absence of the Klf1 protein under nitrogen starvation through gemone-wide analysis using a DNA microarray hybridization technique (**Figure 6**). The over-transcribed genes in the *klf1*Δ mutant encode a variety of proteins with implicated functions in the regulation of the cell wall, nutrient, energy, transport and gene silencing. On the other hand, 9 of the 11 genes transcriptionally suppressed in the mutant were the targets of Ste11, a key transcription factor for sexual development under nitrogen starvation. Another of the group encodes a septin, involved in cytokinesis.

14 ↑ Up-regulated in *klf1Δ*

Gene	Product
SPBC2G2.17c	beta-glucosidase (Psu2)
SPAC869.09	similar to N. crassa conidiation-specific protein 6
SPAC11D3.01c	similar to N. crassa conidiation-specific protein 6
SPCC70.08c	SAM-dependent methyltransferase
ain1	enhancer of RNA-mediated gene silencing
SPBC428.11	homocysteine synthase
SPCC74.04	amino acid permease
SPAC869.01	amidase
SPAC869.06c	cation binding protein
ctr4	copper transporter
SPACUNK4.17	NAD binding dehydrogenase
eno102	enolase
SPAC186.05c	conserved protein, TMEM165
SPBC1348.01	unknown function, DUF999

11 ↓ Down-regulated in *klf1Δ*

Gene	Product
spn6	septin
mam1	ABC transporter
mam2	pheromone p-factor receptor
mam4	protein-s isoprenylcysteine o-methyltransferase
ste6	guanyl-nucleotide exchange factor
rgs1	regulator of G-protein signaling
spk1	serinethreonine protein kinase
ppk33	serinethreonine protein kinase
SPBC4.01	sequence orphan
rec10	involved in meiotic recombination
SPAC5H10.01	conserved protein, DUF1445

Cell wall | Nutrient | Transport | Methylation | Silencing | Energy

Sexual development

Figure 6. Potential target genes of the transcriptional regulation by Klf1. Transcription of these genes are up- or down-regulated in the *klf1Δ mutant* under nitrogen starvation.

GFP (green fluorescent protein)-tagged Klf1 protein mainly localized in the nucleus but was enriched in the non-chromatin region in the interphase of the vegetative cell cycle (**Figure 7**). Under nitrogen starvation, a majority of the GFP-tagged Klf1 protein localized in the chromosomal region.

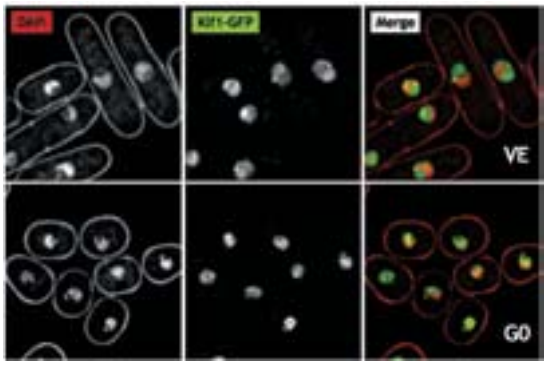


Figure 7. Subcellular localization of the GFP-tagged Klf1.

3.4 The ubiquitin/proteasome system and autophagic pathway guard the cells in G0 phase from the lethal accumulation of oxidative stress

The ubiquitin/proteasome system is engaged in various pathways of cellular regulations, such as cell cycle, quality control of proteins, transcriptional regulation and so on. Metaphase-arrest phenotype caused by a loss-of-function mutation in the 26S proteasome suggested that degrading mitotic regulators (Cyclin and Securin) is one of the essential roles in vegetative proliferation. We previously found that the ubiquitin/proteasome system was also essential and active in G0 phase, however the actual targets and roles are not yet clear.

To explore physiological changes that are induced by proteasome inactivation in G0 phase, we carried out proteomic and metabolomic studies using LC/MS/MS technology on a mutant of a 19S proteome subunit gene, *mts3*. Proteomic analysis revealed that mitochondrial proteins under nitrogen starvation were greatly decreased in *mts3* cells than in wild type cells, but not in vegetative proliferation. (**Figure 8A;** mitochondrial proteins are indicated by red dots). On the other hand, approximately half of the top 20 increased proteins in the *mts3* mutant cells under nitrogen starvation were oxidative stress response proteins. Metabolomic analysis indicated the great increase of cellular anti-oxidants (glutathione and ergothioneine) in the *mts3* and *pts1* (20S proteasome subunit) mutants (**Figure 8B**).

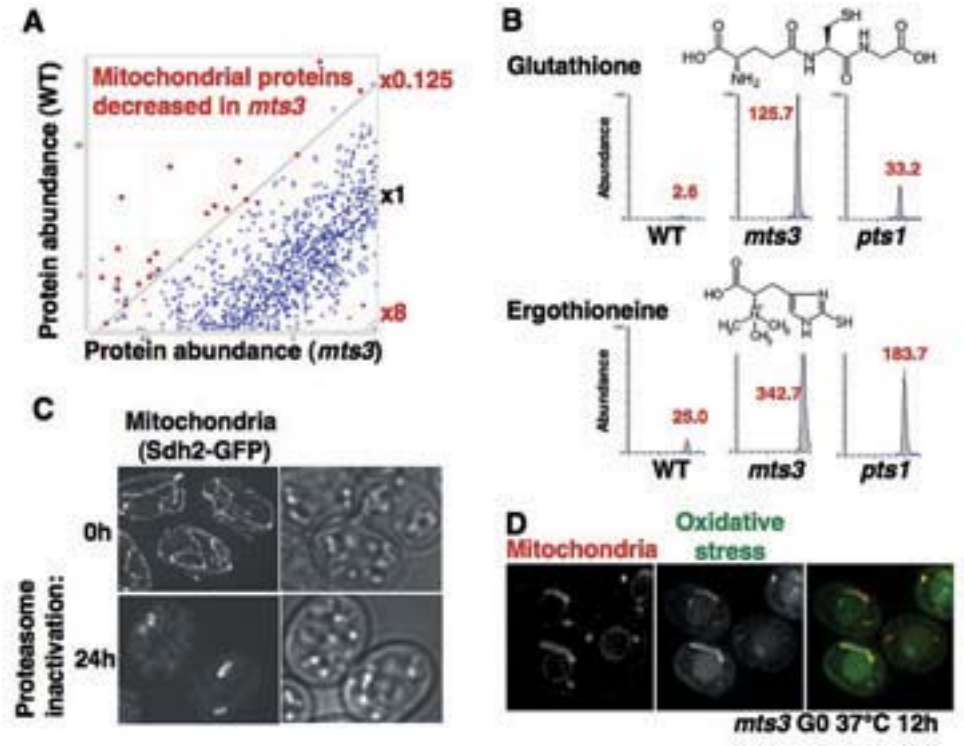


Figure 8. Reduction of mitochondrial proteins and induction of the oxidative stress in the proteasome mutant cells.

Reduction of mitochondria in those mutants under nitrogen starvation was clearly observed by GFP-tagged Sdh2 (succinate dehydrogenase 2), which localized on the inner membrane of mitochondria (**Figure 8C**). Accumulation of oxidative stress was also visualized by staining with H_2DCFDA, an oxidization-sensitive fluorescent dye (**Figure 8D**). Both the nucleus and mitochondria were significantly stained in the mutants under nitrogen starvation. Those results from "-omics" studies and from conventional cell biology consistently show the abnormality of mitochondria and the accumulation of oxidative stress in the cells of proteasome mutants in G0 phase.

Furthermore, we found in the proteasome mutants that the Sdh2-GFP protein was gradually degraded, and that Sdh2-GFP was also incorporated into vacuoles, the equivalent of lysosomes in higher eukaryotes (**Figure 9A**). That degradation was suppressed by PMSF (phenylmethylsulfonyl fluoride), an inhibitor for serine protease in vacuoles. These results suggest that mitochondria are degraded in vacuoles in the proteasome mutant in G0 phase.

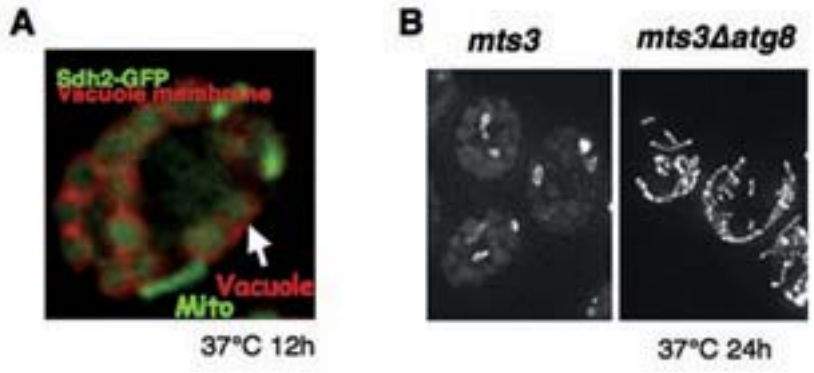


Figure 9. Involvement of vacuoles and the autophagy pathway on the degradation of mitochondria in the proteasome mutant.

Next, we examined the effect of an autophagy mutation (*atg8*Δ) on the degradation of mitochondria and found that inactivation of autophagy completely suppressed the degradation of mitochondria in the proteasome mutant (**Figure 9B**). Selective degradation of mitochondria was suggested because the autophagy reaction was generally active in the wild type cells as well as in the proteasome mutant, though the mechanisms were not clear. That is an important subject to be further elucidated. The autophagy defect caused more severe lethality in proteasome mutant cells with a large accumulation of oxidative stress, and additionally, that lethality could be prevented by N-acetyl cysteine, an antioxidant (**Figure 10**).

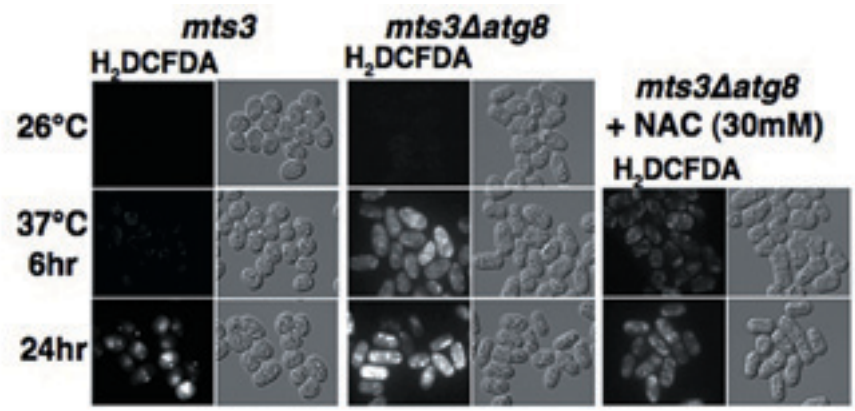


Figure 10. NAC, an anti-oxidant suppressed the lethality caused from defective autophagy in the proteasome mutant.

Thus, it is suggested that the lethal accumulation of oxidative stress is reduced by an autophagy-dependent selective degradation of mitochondria, the major source of active oxygen in the cell (Fig. Y D). The detailed mechanism of this self-maintenance pathway is to be elucidated.

3.5 A drug-using approach to find essential cellular activities for maintaining viability of G0 cells

It has been generally thought that proliferating cells are more sensitive to drugs than differentiated, non-dividing cells, because inhibition of the steps in DNA replication or mitosis cause severe irreversible damage to cell viability. On the contrary, if we find a drug that reduces the viability of non-dividing cells, it could be a clue to reveal essential activities in the non-dividing cells.

We previously found 8 drugs that can cause loss of viability through the screening 95 drugs in the SCADS inhibitor kit (a gift from the Screening Committee of Anticancer Drugs supported by a Grant-in-Aid for Scientific Research on Priority Area "Cancer" from the Ministry of Education, Culture, Sports, Science, and Technology, Japan). Notably, Tamoxifen shows more toxicity to non-dividing G0 cells than to vegetatively proliferating cells. We found that low concentrations of Tamoxifen (10 μM, which does not cause fetal damage) affected the membrane structure of the cell, suggesting the activation of endocytosis (**Figure 11**).

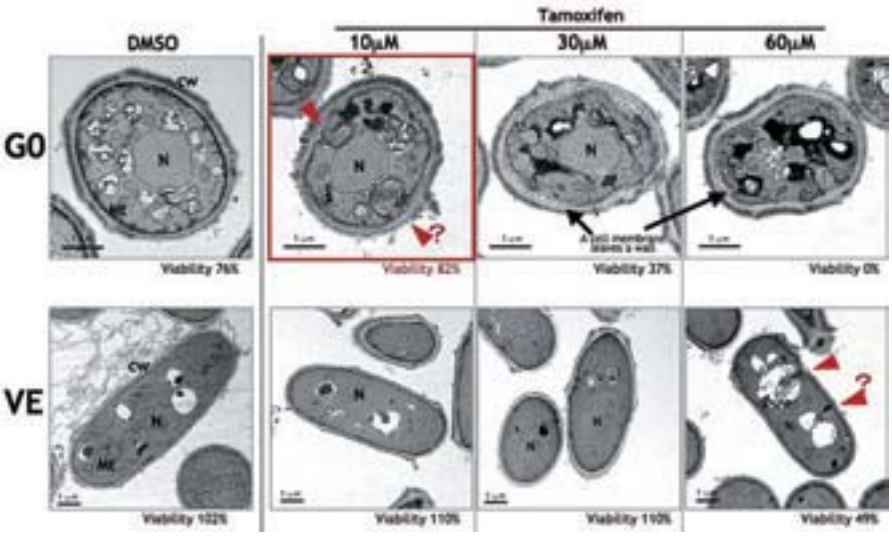


Figure 11. Effects of tamoxifen on fission yeast cells in the G0 phase and in vegetative proliferation.

We screened a haploid gene deletion mutant set for Tamoxifen-supersensitive or resistant mutants and found a resistant mutant of (*pzh1*) gene, which encodes a protein phosphatase (**Figure 12**). We have not yet isolated a supersensitive mutant.

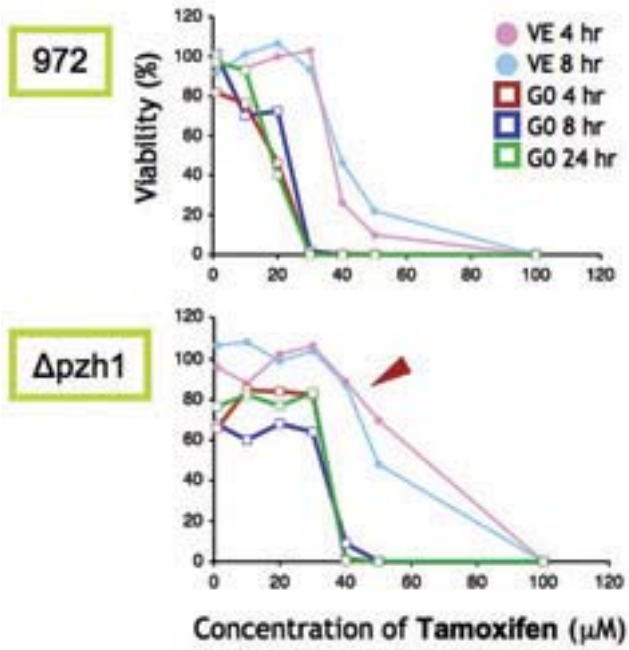


Figure 12. *pzh1Δ* mutant is resistant to tamoxifen in the vegetative phase.

3.6 Metabolic profiling of *S. pombe* cells under different environmental and genetic conditions

We further improved our metabolic profiling protocol utilizing ZIC-pHILIC (zwitterionic column for Hydrophilic Interaction Liquid Chromatography) separation and mass spectrometric detection using LTQ Orbitrap mass spectrometer. Approximately 2700 peaks and 3400 peaks could be detected and quantified in negative ionization mode and in positive ionization mode, respectively. Among these, approximately 200 metabolites could be identified so far, most of which we verified using pure standards. We applied this method to comparisons of wild type proliferating and nitrogen-starved G0 cells (**Figure 13**).

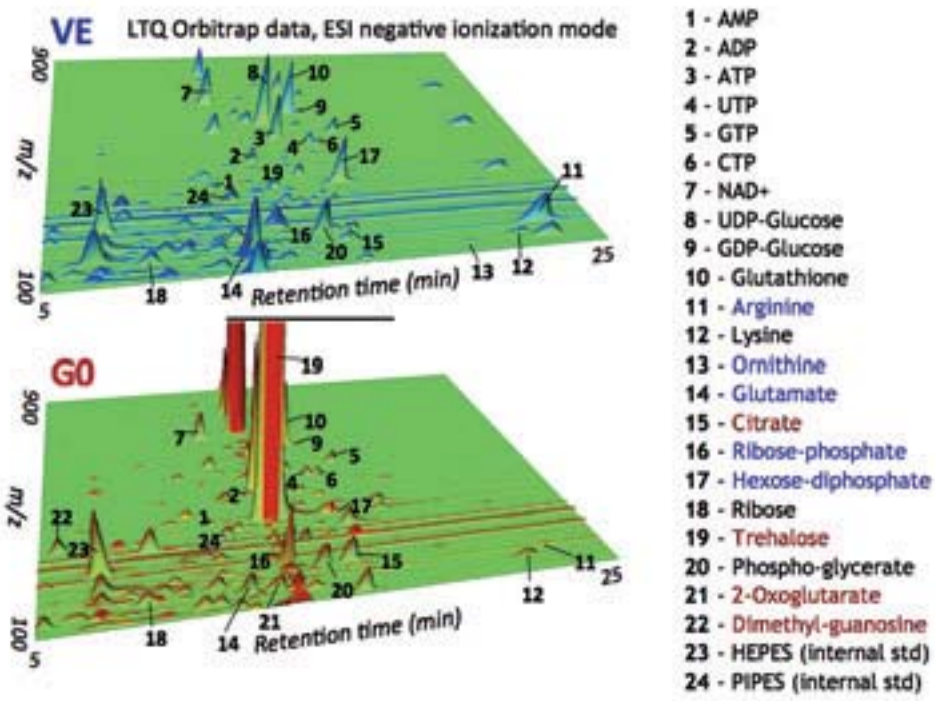


Figure 13. 3D LC-MS metabolic profiles of vegetative (VE) and G0 cells

Taking advantage of powerful yeast genetics, we further analyzed the metabolome of several mutant strains. The *tor2-287* temperature-sensitive mutant, defective in nutrient sensing and mimicking the G0 state at restrictive temperature, showed several major metabolic effects, namely overproduction of amino acids, an increase of inosine and depletion of ribulose. Similar results were observed when this mutant was treated with rapamycin at a permissive temperature. Analysis of MAP kinase mutants *wis1* and *spc1*, defective in G0 entry, confirmed their inability to adapt to nitrogen-lacking medium, as G0-specific metabolic changes could not be observed in these mutants. Subtle alteration of the metabolome was found in ferrichrome synthetase deletion mutant *sib1*Δ, where disappearance of ferrichrome and appearance of putative precursor dipeptide had little effect on the overall metabolic profile. On the contrary, analysis of the HMG-CoA synthase mutant *hcs1-143* revealed extensive alterations in various areas of metabolism, including urea cycle intermediates and several acetylated compounds (**Figure 14**). This confirmed the importance of HMG-CoA, a key metabolite of the highly conserved Mevalonate pathway, for various cellular processes, despite the apparent low abundance of free HMG-CoA within cells. Profiling of a temperature sensitive proteasome mutant (*mts3*) revealed the increase of several compounds related to oxidative stress in this mutant at G0 state at a restrictive temperature.

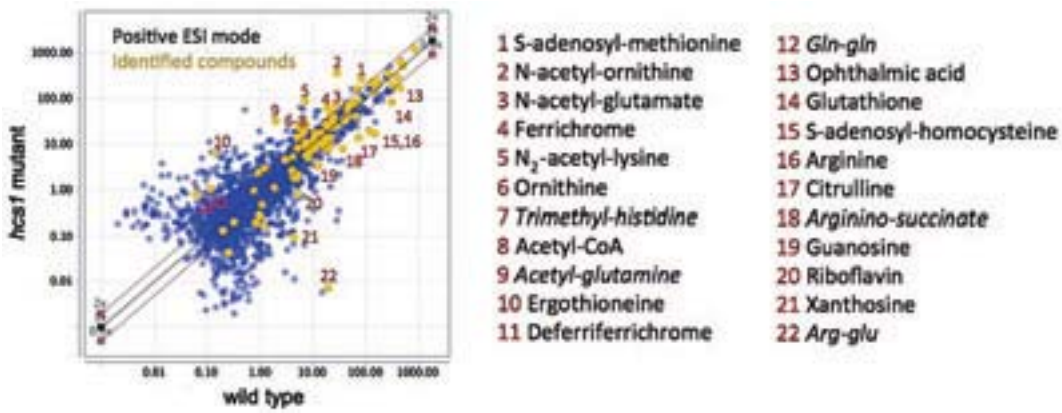


Figure 14. Metabolomic comparison of exponentially growing wild type and *hcs1-143* ts mutant cells visualized using a scatter plot generated by MZviewer software. Identified metabolites are indicated by yellow color.

A software system called MZviewer was developed in order to visualize the semi-quantitative LC-MS data sets in a dynamically generated scatter plot (**Figure 8A**). This allowed us to perform quick comparisons of thousands of peaks between different samples. Also a standalone program was developed to visualize transcriptomic and proteomic data (BolomView). Both use scatter plotting as a graphic tool to visualize the strength and the direction of the relationship between the variables. One of the most powerful aspects of a scatter plot is its ability to show nonlinear relationships between variables. Furthermore, if the data is represented by a mixture model of simple relationships, these relationships will be visually evident as superimposed patterns. As part of this tool a histogram pot is available to verify the distribution of the data.

4 Publications

4.1 Journals

Adachi, Y., Kokubu, A., Ebe, M., Nagao, K., & Yanagida, M. Cut1/separase-dependent roles of multiple phosphorylation of fission yeast cohesion subunit Rad21 in post-replicative damage repair and mitosis. *Cell cycle (Georgetown, Tex.)* 7 (6), 765-776 (2008).

Nakazawa, N., Nakamura, T., Kokubu, A., Ebe, M., Nagao, K., & Yanagida, M. Dissection of the essential steps for condensin accumulation at kinetochores and rDNAs during fission yeast mitosis. *The Journal of cell biology* 180 (6), 1115-1131 (2008).

Hanyu, Y., Imai, K.K., Kawasaki, Y., Nakamura, T., Nakaseko, Y., Nagao, K., Kokubu, A., Ebe, M., Fujisawa, A., Hayashi, T., Obuse, C., & Yanagida, M. Schizosaccharomyces pombe cell division cycle under limited glucose requires Ssp1 kinase, the putative CaMKK, and Sds23, a PP2A-related phosphatase inhibitor. *Genes Cells* (2009).

Sajiki, K., Hatanaka, M., Nakamura, T., Takeda, K., Shimanuki, M., Yoshida, T., Hanyu, Y., Hayashi, T., Nakaseko, Y., & Yanagida, M. Genetic control of cellular quiescence in S. pombe. *J Cell Sci* 122 (Pt 9), 1418-1429 (2009).

4.2 Book(s) and other one-time publications

Yanagida, M. The Kinetochore: From Molecular Discoveries to Cancer Therapy, Chapter2, "The Basics of Chromosome Segregation", *Springer Science+Business Media*, 21-44 2008

4.3 Oral presentations

Yanagida, M. Genes networks for the cell survival under nutritional stress in fission yeast, Institute de Biologie de Lille, Lille, France, April 25, 2008

Takeda, K. The selective degradation of mitochondria is induced by ubiquitin/proteasome dysfunction in fission yeast G0 phase., National Institute for Basic Biology, Okazaki, Japan, April 25, 2008

Yanagida, M. DNA replication and chromosome cohesion, The Cell Cycle and Genomic Stability, Conferences Jacques-Monod, Roscoff, France, April 26-30, 2008

Yanagida, M., Control of Mitosis and Centromere/Kinetochore Formation, International Symposium on Chromosome Dynamics, Mie, Japan, May 28-30, 2008

Yanagida, M. Fission yeast, which never fission, 18th Yeast Symposium, Kobe, Japan, June 5, 2008

Yanagida, M. Transcriptomic, Proteomic and Metabolomic analyses on entry, maintenance and exit mechanism of fission yeast G0 phase, 6th Life surveyor symposium, Osaka Japan, June 26, 2008

Yanagida, M. Chromosome Dynamics and Centromere / Kinetochore Formation in the cell cycle, IRIC Seminar, University of Montreal, Montreal, Canada, July 18, 2008

Yanagida, M. Lee Hartwell Lecture, Yeast 2008 Genetics and Molecular Biology Meeting, University of Toronto, Toronto, Canada, July 22-26, 2008

Yanagida, M. Centromeres and Kinetochores in Fission Yeast and Human Cells, CHROMOSOME SEGREGATION:Centromeres & Kinetochores Meeting, Palais des Congres, Arcachon, Bordeaux, France, Sept.27-Oct.2, 2008

Pluskal, T. Metabolomic profiling of cellular quiescence, LTQ Orbitrap User Meeting, Thermo Fisher Scientific, Yokohama, Japan, November 13, 2008

Yanagida, M. Difference between dividing- and non-dividing cells: from a view of metabolism, 5th Kyoto cardiovascular metabolism seminar, Osaka, Japan, December 1, 2008

Yanagida, M. Functional Analysis of Condensin Complex, The 31st Annual Meeting of the Molecular Biology Society of Japan, Kobe, Japan, December 9-12, 2008

Hayashi, T., Hatanaka, M., Nagao, K., Nakaseko, Y., Kanoh, J., Kokubu, A., Ebe, M., Fujisawa, A., Fujita, Y., Ikai, N., Yanagida, M. TOR complexes and rapamycin sensitivity in fission yeast, The 31st Annual Meeting of the Molecular Biology Society of Japan, Kobe, Japan, December 9-12, 2008

Shimanuki, M., Uehara, L., Pluskal, T., Kokubu, A., Nagao, K., Yanagida, M. Genes required for maintaining cell viability in G0 phase, The 31st Annual Meeting of the Molecular Biology Society of Japan, Kobe, Japan, December 9-12, 2008

Takeda, K., Kikukchi, S., Nagao, K., Kokubu, A., Yoshida, T., Yanagida, M. The quality control of mitochondria is achieved by Ub/Proteasome and autophagy in fission yeast G0 phase., The 31st Annual Meeting of the Molecular Biology Society of Japan, Kobe, Japan, December 9-12, 2008

Takeda, K., Kikukchi, S., Nagao, K., Kokubu, A., Yoshida, T., Yanagida, M. The quality control of mitochondria is achieved by Ub/Proteasome and autophagy in fission yeast G0 phase., 8th J-mit, Tokyo, Japan, December 18, 2008

Shimanuki, M., Uehara, L., Pluskal, T., Kokubu, A., Nagao, K., Yanagida, M. Fission yeast Klf1 is involved in the maintenance of the cells in the G0 phase., The 26th Chromosome Workshop, Himeji, Japan, January 26-28, 2009

Yanagida, M. Difference between dividing- and non-dividing cells: from a view of metabolism, 1st NAGOYA Global retreat, Nagoya, Japan, February 20, 2009

4.4 Posters

Hayashi, T., Hatanaka, M., Nagao, K., Nakaseko, Y., Kanoh, J. Nutrient-sensing TORC and Damage-responsive Rad3/ATR are linked by Tel2 and Tti1 complex that associate with all of the PIK-related kinases, Ataxia-Telangiectasia Workshop 2008, Otsu, Japan, Apr 22-26, 2008

Takeda, K., Kikuchi, S., Nagao, K., Kokubu, A., Yoshida, T., Yanagida, M. The selective degradation of mitochondria is induced by ubiquitin/proteasome dysfunction in fission yeast G0 phase, EUROMIT7, Stockholm, Sweden, June 11-14, 2008

Pluskal, T., Nagao, K., Nakamura, T., Villar Briones, A., Yanagida, M. High-resolution LC-MS analysis of S. pombe intracellular metabolome, 5th International Conference on Plant Metabolomics, Yokohama, July 15-18, 2008

Hayashi, T., Fujita, Y., Ebe, M., Nagao, K., Kokubu, A. Identification of Novel Centromere Proteins Mis19 and Mis20 as Mis18 Interacting Partners in Fission Yeast, EMBO Workshop, Chromosome Segregation: Centromeres & Kinetochores, Arcachon, Bordeaux, France, Sep. 27- Oct. 2, 2008

Takeda, K., Yanagida, M. The'double-lock' system for the quality control of mitochondria in the G0 quiescent phase is achieved by UPS and autophagy, Protein Community, Okinawa, Japan, November 23-26, 2008

Shimanuki, M., Uehara, L., Pluskal, T., Kokubu, A., Nagao, K., Yanagida, M. Genes required for maintaining cell viability in G0 phase., The 31st annual meeting of the Molecular Biology Society of Japan, Kobe, Japan, December 9-12, 2008

Takeda, K., Kikukchi, S., Nagao, K., Kokubu, A., Yoshida, T., Yanagida, M. The quality control of mitochondria is achieved by Ub/Proteasome and autophagy in fission yeast G0 phase, The 31st annual meeting of the Molecular Biology Society of Japan, Kobe, Japan, December 9-12, 2008

Takeda, K. The quality control of mitochondria is achieved by Ub/Proteasome and autophagy in fission yeast G0 phase, 8th J-mit, Tokyo, Japan, December 18th, 2008

5 Intellectual Property Rights and Other Specific Achievements

Nothing to report.

6 Meetings and Events

6.1 The 3rd International Workshop on Cell Regulations in Division and Arrest Under Stress

Date: April 6-10, 2008
Venue: OIST Seaside House
Speakers: Mohan Balasubramanian (The Nat. Univ. of Singapore)
Monika Gullerova (Univ. of Oxford)
Isabelle Jourdain (Massey Univ.)
Andrea Musacchio (European Institute of Oncology)
David Pellman (Harvard Medical School)
Janni Petersen (Univ. of Manchester)
Jonathon Pines (Wellcome Trust / Cancer Research UK)
Kazuhiro Shiozaki (UC Davis)
Tim Stearns (Stanford Univ.)
Hiroyuki Takai (Rockefeller Univ.)
Atsushi Miyawaki (RIKEN Brain Science Institute)
Atsushi Hirao (Kanazawa University)
Shunsuke Ishii (Tsukuba, RIKEN)
Kazuhiro Iwai (Osaka City Univ.)
Takashi Kadowaki (Univ. of Tokyo)
Yoshiaki Kamada (Nat. Inst. for Basic Biology)
Junko Kanoh (Kyoto Univ.)
Shigeaki Kato (Univ. of Tokyo)
Tatsuhiko Kodama (Univ. of Tokyo)
Tomohiro Matsumoto (Kyoto Univ.)
Masayuki Miura (Univ. of Tokyo)
Noboru Mizushima (Tokyo Metrop. Inst. of Med. Sc.)
Katsutoshi Mori (Kyoto Univ.)
Keiichi Nakayama (Kyushu Univ.)
Keiko Nakayama (Tohoku Univ.)
Masayuki Yamamoto (Tohoku Univ.)
Shinya Yamanaka (Kyoto Univ.)
Mitsuhiro Yanagida (OIST, Kyoto Univ.)
16 posters were also presented by the participants

6.2 OIST seminar "Mitophagy, degradation of mitochondria via an autophagy-related pathway in budding yeast"

Date: November 26, 2008
Venue: OIST IRP seminar room
Speaker: Dr. Koji Okamoto (National Institute of Basic Biology, JAPAN)

Education and Training Activities

During fiscal year 2008, 13 workshops either hosted or co-sponsored by OIST took place, brining as many as 570 lecturers and participants together for vigorous scientific discussions.

The 3rd International Workshop on Cell Regulations in Division and Arrest Under Stress

Outline : The featured topic of the third International Workshop on Cell Regulations in Division and Arrest under Stress was stress response with respect to the regulation of cell division and arrest. The workshop provided an outstanding opportunity for all participants to exchange new ideas and enjoy exciting discussions.

Date : April 6 –10, 2008
Organizer : Dr. Mitsuhiro Yanagida, OIST
Venue : OIST Seaside House
Participants : Lecturers 28, Participants 38

Protein 3D Structure Visualization and Structural Bioinformatics Workshop

Outline : The course, designed for faculty, post-doctoral researchers, research staff and graduate students in biochemical sciences, involved participants to use highly user-friendly software for visual investigation of 3D structures of proteins, nucleic acids, and their interactions with each other, as well as with ligands, substrates, and drugs, and protein evolutionary conservation. Hands-on experience largely with molecules of each participant's choice was also explored.

Date : May 13, 2008
Organizer : Dr. Fadel Samatey, OIST
Venue : OIST Research Laboratory
Participants : Lecturer 1, Participants 16

G8 Science and Technology Ministerial Satellite Workshop on Environment and Energy Issues in Okinawa

Outline : As part of satellite activities for the first Group of Eight (G8) Ministerial-Level Meeting on Science and Technology on June 15, 2008 in Okinawa, a workshop on environment and energy issues took place on June 14, 2008 at the University of the Ryukyus. In the workshop, four guest speakers each delivered a lecture and participated in a Q&A session including OIST President, Dr. Sydney Brenner, BOG members Dr. Akito Arima, Dr. Steven Chu, Dr. Yuan Tseh Lee, and Special Advisor Dr. Hiroaki Kitano.

Date : June 14, 2008
Organizers : OIST P.C., The University of the Ryukyus, Academia Sinica, Lawrence Berkeley National Laboratory
Supporter : Okinawa Prefecture
Venue : The University of the Ryukyus
Participants : Lecturers 4, Participants 250

Okinawa Computational Neuroscience Course (OCNC) 2008

Outline : The aim of OCNC 2008 was to provide opportunities for young researchers with theoretical backgrounds to learn up-to-date neurobiological findings, and those with experimental backgrounds to have hands-on experience in computational modeling.

Date : June 16 – July 3, 2008
Organizers : Dr. Erik De Schutter, OIST
Dr. Kenji Doya ,OIST
Dr. Klaus Stiefel, OIST
Dr. Jeff Wickens, OIST
Venue : OIST Seaside House
Participants : Lecturers 19, Tutors 7, Participants 31

Workshop on Open Problems in the Neuroscience of Decision Making

Outline : During the last decade, the study of "decision making" outgrew the domains of philosophy and economics into major subjects of neuroscience and machine learning. This workshop focused on four outstanding issues in the neural mechanisms of decision making.

Date : October 16-18, 2008
Organizers : Dr. Kenji Doya, OIST et al.
Venue : OIST Seaside House
Participants : Lecturers 16, Participants 21

Multi-Scale Phenomena in Biology Workshop

Outline : A multitude of biological phenomena are described at multiple levels. The workshop delved into the commonalities and differences between neuroscience, evolutionary biology, molecular biology and ecology in this regard. It also looked at how mathematics can help in describing these phenomena.

Date : November 4-6, 2008
Organizers : Dr. Robert Sinclair, OIST
Dr. Klaus Stiefel, OIST
Venue : OIST Seaside House
Participants : Lecturers 11, Participants 24

Workshop on Gradients and Signalling: From Chemotaxis to Development

Outline : The workshop included sessions on bacterial chemotaxis and signaling by growth factors, TGF-beta, Hedgehog and Wnt. The aim of the meeting was to discuss the formation of gradients as well as the intracellular signaling pathways that receive them. Discussion took place on the similarities and differences in mechanisms a variety of signalling pathway utilize to create changes in target cells.

Date : November 17-21, 2008
Organizers : Dr. Ichiro Maruyama, OIST
Dr. Ichiro Masai, OIST
Dr. Mary Ann Price, OIST
Dr. Fadel Samatey, OIST
Venue : OIST Seaside House
Participants : Lecturers 25, Participants 37

Winter Course "Evolution of Complex Systems"

Outline : The aim of the course was to provide opportunities for young researchers with biological backgrounds to learn the latest advances in the field of evolutionary developmental biology, and for those with theoretical backgrounds to have hands-on experience in gene expression.

Date : December 8-14, 2008
Organizers : Dr. Sydney Brenner, OIST
Dr. Noriyuki Satoh, OIST
Dr. Ichiro Masai, OIST
Dr. Mary Ann Price, OIST
Venue : OIST Seaside House
Participants : Lecturers 6, Tutors 8, Participants 21, Observers 6

Co-sponsored Workshop

Workshop "Mechanism of Brain and Mind" on August 9-10, 2008 in Sapporo, Hokkaido
Workshop "Mechanism of Brain and Mind" on January 13-15, 2009 in Rusutsu Resort, Hokkaido
Workshop "Mathematical Modeling and Problem Solving" on March 5-6, 2009 at OIST Seaside House
Workshop "Neural and Brain Systems Team Meeting" on March 8-9, 2009 at OIST Seaside House
Workshop "BioHackathon 2009" on March 15-21, 2009 at OIST Seaside House

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