Department of Integrative Physiology

Outline and Research Objectives

This department was initially established in 1877 as the First Department of Physiology, and reorganized in 1997 as the Department of Integrative Physiology. Our department collaborates with other laboratories dedicated to Physiological Sciences, that is, the Department of Molecular/Cellular Physiology and the Department of Neurophysiology, in teaching activities for undergraduate courses and the nursing school. The fields in which our department specializes include the entire spectrum of the physiology of "animal functions", such as general physiology, sensory physiology, neurophysiology, higher nervous functions and cognitive neurosciences.

Faculties and Students

Professor and Chair	Yasushi Miyashita, Ph.D. (1989-)
Lecturer	Isao Hasegawa, M.D., Ph.D. (2000-)
Associate	2
Postdoctoral Fellow	5
Graduate Student	10
Research Student	1
Secretary	3

Past Research and Major Accomplishments

Most of our research has been focused on the higher brain functions of the mammalian central nervous system, in particular, the neural mechanisms of cognitive memory in the primate. The basic motivation of the research can be best explained as follows:

Knowledge or experiences are voluntarily recalled from memory by reactivation of their neural representations in the cerebral association cortex. Three questions are central in the understanding of this process:

(1) Where are mnemonic representations coded and how are they organized?
(2) Which neural processes create the representation?
(3) What is the mechanism underlying reactivation of the representation on demand of voluntary recall?

1. Creating the mnemonic representation

Lesion studies in primates have implicated the IT cortex in long-term memory storage of visual objects [40]. Neuronal correlates of associative long-term memory were first discovered in the IT cortex by Miyashita [49, 50] and Sakai & Miyashita [45]. He developed a novel memory paradigm that requires the subject to create a link of associative memory between mathematically designed pictures (for examples, see Fig.1). Their single-unit recording experiments in thepair-association task identified two mnemonic properties of IT neurons. First, the stimulus-selectivity of IT neurons can be acquired through learning in adulthood. Second, the activity of IT neurons can link the representations of temporally associated but geometrically unrelated stimuli.

2. Activating the representation in the temporal cortex on demand

In spite of the classical clinical observation that electric stimulation of the temporal lobe produces 'experiential responses', there have been no direct evidence supporting the notion of 'reactivation of neural representations' during memory retrieval. We first reported a neuronal correlate of the reactivation process as 'pair-recall neurons' [42, 45]. In our subsequent study [30], we devised a new modified pair-associate task in which the necessity for memory retrieval and its initiation time were controlled by a colour switch, independent of the cue stimulus presentation. Single-unit recordings in monkeys performing this task revealed that IT neurons selective to a memorized object are dynamically activated at the time of memory retrieval of that object, and suppressed at the time of retrieval of other objects. Then, it became important to determine the neural network that drives the memory retrieval machinery in the IT cortex.

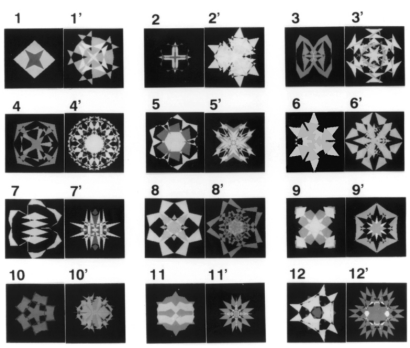

Figure 1 : Paired associates. Each pictures was created according to a fractal algorithm.

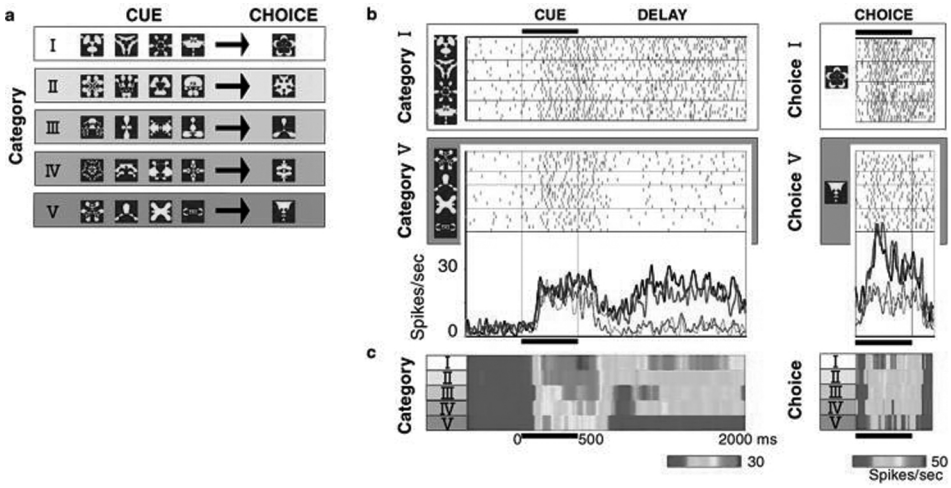

Figure 2 : Which information is carried via top-down signals? (a) A stimulus-stimulus association task. Twenty cue-pictures are randomly sorted into five categories. Each of the four cues in one category specifies a common choice. (b) Category-selective delay activity of an inferior temporal neuron. Delay activities were raised for all cues in Category I, but not for any cues in Category V (rastergrams, top-down condition). Choice responses are also strongest for Category I and weakest for Category V. Spike density functions show averaged activities across four cues in Category I (thick) and in Category V (thin) for both conditions (top-down, blue; bottom-up, black). (c) Spike density functions of the top-down response for five categories, as well as the choice responses, shown by a pseudo-colour coding. Note that the category-selective delay activity can predict choice selection.

3. Top-down activation through fronto-temporal pathway

A candidate component of the neural circuit is the top-down activation from the prefrontal cortex. The prefrontal cortex has been implicated in various executive processes, and its contribution to mnemonic functions, particularly in episodic memory and working memory, is repeatedly demonstrated in human neuroimaging studies. We attempted to directly test its contribution to memory retrieval control by the capacity for interhemispheric transfer of mnemonic signal through the anterior corpus callosum, a key structure interconnecting prefrontal cortices. We introduced the posterior-split-brain paradigm into the associative memory task in monkeys [23]. Long-term memory acquired through stimulus-stimulus association did not interhemispherically transfer via the anterior corpus callosum. Nonetheless, when a visual cue was presented to one hemisphere, the anterior callosum could instruct the other hemisphere to retrieve the correct stimulus specified by the cue. Therefore, although visual long-term memory is stored in the temporal cortex, memory retrieval is under the executive control of the prefrontal cortex.

In spite of predictions based on these behavioural experiments, no neuronal correlate of the top-down signal from the prefrontal cortex to IT cortex had been detected. We provided the first evidence of the existence of the top-down signal [14] by conducting single-unit recording in posterior-split-brain monkeys. In the absence of bottom-up visual inputs, single IT neurons were robustly activated by the top-down signal, which conveyed information on semantic categorization imposed by visual stimulus-stimulus association (Fig. 2). We also demonstrated that the top-down signal had a longer latency by about 100 ms than the bottom-up signal. The longer latency is most likely ascribed to a multi-synaptic conduction delay that reflected the signal transformation within the prefrontal cortices.

Current Research and Future Prospects

Most of our current research is focused on the third question on the mechanisms of cognitive memory, that is, (3) What is the mechanism underlying reactivation of the representation on demand of voluntary recall?

Two complementary approaches are of interest in answering the above question. First, neuroimaging studies in humans can elucidate brain representations and their interactions in high-level memory processes, which have been conceptualized as retrieval attempt or contextual monitoring. Second, the well-analyzed neural representations in the monkey cortex should be compared with those in the human cortex. The comparison would provide strong evidence that couples active neural codes in the brain network with conscious experiences.

An example of our recent achievements along the first approach is the discovery of a neural correlate of the "feeling-of-knowing" [1]. The "feeling-of-knowing (FOK)" is a subjective sense of knowing a word before recalling it, and it provides us clues to understanding the mechanisms of human meta-memory systems. We investigated neural correlates for the FOK based on the recall-judgment-recognition paradigm. Event-related functional magnetic resonance imaging (fMRI) with parametric analysis was used. We found activations in the left dorsolateral, left anterior, bilateral inferior, and medial prefrontal cortices that signifi-

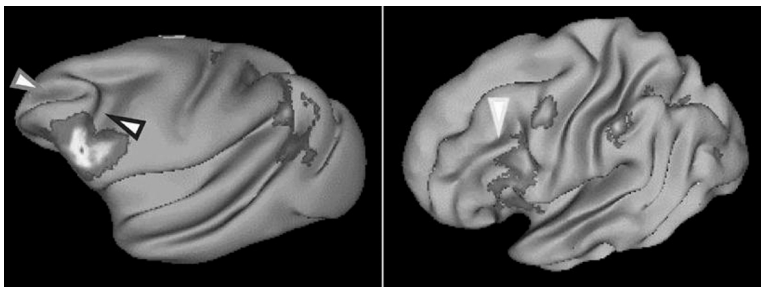

Figure 3 : Comparison of activated cortical areas in set-shifting of WCST in monkeys (left) and humans (right).

cantly increased as the FOK became greater. Furthermore, we demonstrated that the FOK-region in the right inferior frontal gyrus and a subset of the FOK-region in the left inferior frontal gyrus are not recruited for successful recall processes, suggesting their particular role in meta-memory processing.

Along the second approach, we have recently demonstrated that fMRI studies in macaque monkeys allow us to directly compare the brain activity of humans with that of monkeys with respect to high-level cognitive functions [2]. In this study, the functional brain organization of macaque monkeys and humans was directly compared by fMRI. Subjects of both species performed a modified Wisconsin Card Sorting Test that required behavioural flexibility in the form of cognitive set shifting. Equivalent visual stimuli and task sequence were used for the two species. We found transient activation related to cognitive set shifting in focal regions of the prefrontal cortex in both monkeys and humans. These functional homologs were located in cytoarchitectonically equivalent regions in the posterior part of the ventrolateral prefrontal cortex. This comparative imaging provides insights into the evolution of cognition in primates.

Various neuroimaging studies have been carried out to clarify cognitive functions of the human brain. Because most neuroimaging studies rely on the correlation between cognitive processes and brain activations, conjunction with other complementary methods, such as neuropsychological studies and transcranial magnetic stimulation, should be promoted to clarify the behavioural significance of observed brain activities. Emerging fMRI of the monkey brain enables us to directly compare the brain activity of humans with that of monkeys using the same modality. Therefore the monkey fMRI will make it possible to combine imaging studies with electrophysiology or lesion studies, and will hopefully lead to the understanding of the causal relationship between activated brain areas and cognitive functions. Since most of the detailed knowledge of anatomy and function of the cerebral cortex has come from studies in monkeys, sharing the same method among the studies of monkeys and humans would advance the understanding of the neural basis of human cognition.

Research Grants

1. A Grant-in-Ai32d for Specially Promoted Research, MEXT (07102006)
   Neural mechanisms of cognitive memory system : Integration of functional magnetic resonance imaging method and molecular/cellular approaches.
   ¥ 215,000,000 (1995-1999)
2. A Grant-in-Aid for Specially Promoted Research, MEXT (Extension 07102006)
   Neural mechanisms of cognitive memory system : Integration of functional magnetic resonance imaging method and molecular/cellular approaches.
   ¥309,000,000 (2000-2001)
3. A Grant-in-Aid for Specially Promoted Research, MEXT (14002005)
   Brain distributed network : Integrative study based on functional MRI in monkeys
   ¥ 550,000,000 (2002-2006)

Select Publications

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Department of Cellular and Molecular Physiology

Outline and Research Objectives

Knowledge comes to man through the door of the senses (Heraclitus). Using multidisciplinary approaches including electrophysiology, optical imaging, molecular / cell biology, and molecular genetics, we aim at better understanding of neuronal mechanisms for the sensory perception of the external world and for the emotional state induced in the brain by the sensory inputs. For this purpose we are currently analyzing the central nervous system for olfaction, a sensory modality that has a strong influence to human emotion. Another major research focus of this department is to understand cellular and molecular mechanisms for contact-mediated interactions between neurons and immune cells that occur in pathological and physiological conditions.

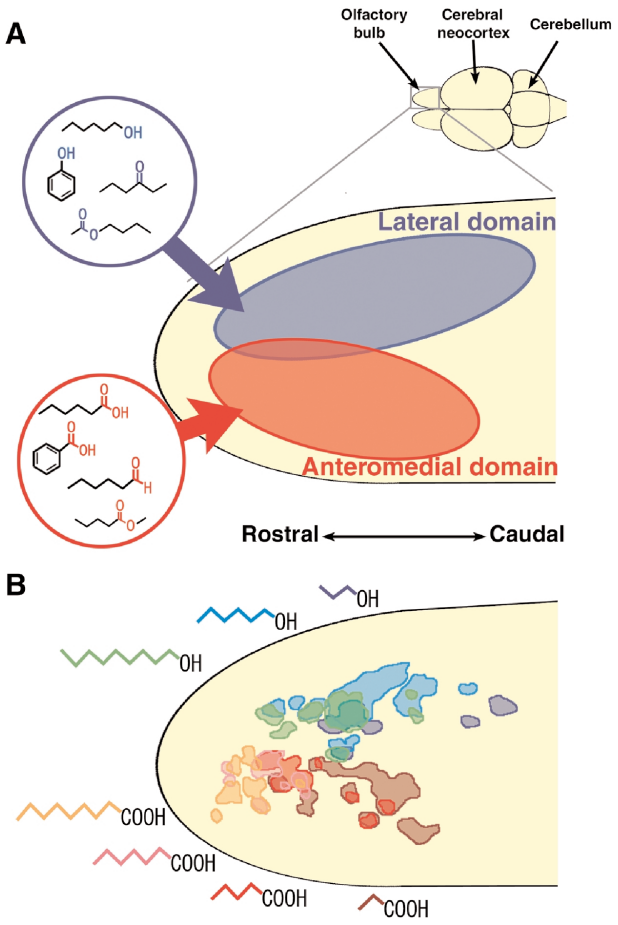
Faculties and Students

Professor and Chair	Kensaku Mori, Ph. D. (May 1999 ~)
Lecturer	Masahiro Yamaguchi, M.D., Ph. D.
Associate	2
Graduate student	12
Research student	1
Secretary	1

Past Research and Major Accomplishments

K. Mori and colleagues have been studying the functional organization of the olfactory nervous system for nearly 28 years. Major accomplishment of our research group before 1999 includes (1) a systematic functional and structural analysis of the neuronal circuits in the mammalian olfactory bulb, the first center for the processing of olfactory information in the brain, (2) the finding that individual principal neurons in the olfactory bulb respond selectively to a range of odorants having common molecular structures, and (3) the finding of zone-to-zone axonal connectivity pattern between olfactory sensory epithelium and the olfactory bulb. After K. Mori's arrival at the Department of Cellular and Molecular Physiology in May 1999, we further extended our previous studies and found a clue for understanding the spatial organization of the 'odor map' in the olfactory bulb (Fig.1; e. g., Mori et al., Science, 1999, Uchida et al., Nature Neurosci., 2000., Nagao et al., NeuroReport, 2000). These studies as well as our novel molecular genetic approaches (Yoshihara et al., Neuron 1999) formed a basis for current and future studies on the 'odor maps' in the olfactory bulb and olfactory cortex (e.g., Inaki et al., Eur. J. of Neurosci., 2002).

We previously found several novel neuronal cell adhesion molecules and analyzed their functional roles in the formation of neuronal circuit in the brain (e.g., telencephalin (TLCN), Yoshihara et al., Neuron, 1994; OCAM, Yoshihara et al., J. Neurosci., 1997). We are currently extending the analysis of the functional roles of these cell adhesion molecules using TLCN-deficient mice (in collaboration with Prof. Mishina) and OCAM-deficient mice. During the course of the analysis of TLCN, we unexpectedly found that the binding between TLCN and its counterreceptor LFA-1 mediated the interaction between neurons and



Domains in the odor map of the olfactory bulb

immune cells (Mizuno et al., J. B. C., 1997; Tian et al., J. Cell Biol., 2000, Eur. J. Immunol., 2000, J. Immunol., 1997). We thus developed a co-culture assay system to examine the contact-mediated interactions between neurons and immune cells.

Our group generated transgenic mice in which newly generated neurons are visualized in the brain by the green fluorescent protein (Yamaguchi et al., NeuroReport, 2000). Using the transgenic mice, we are now studying the neurogenesis and neuron-elimination in the adult brain.

Current Research

Current research programs and targets can be categorized into the following four topics.

(1) Functional analysis of the neuronal circuits in the central olfactory nervous system

An astonishing feature of the olfactory system is its ability to distinguish among more than 400,000 different odorants and among countless number of odorants-mixtures, each having specific 'odor'. Using optical imaging and electrophysiological methods, we are studying the 'odor maps' in the central olfactory system to understand the logic employed by the olfactory system for discrimination among numerous odorants and for perception and learning of the olfactory image of objects. The target regions for the mapping include the olfactory bulb and several regions of the olfactory cortex (e.g., piriform cortex and olfactory tubercle). In addition, odorants having pleasant or unpleasant 'odor' are used to map the brain regions and to elucidate neuronal mechanisms responsible for the emotional states of the brain.

(2) Neurogenesis and neuron-elimination in the adult brain

Olfactory nervous system has an unusual capacity to generate neurons throughout life. The sensory neurons in the nasal epithelium and the local interneurons in the olfactory bulb are turning over continuously even in the adult. Therefore, we choose the olfactory system as a model system with which to study the molecular and cellular mechanisms for the recruitment of newly generated neurons into the adult central nervous system and for the selective elimination of damaged neurons from the functional neuronal circuit. Molecular-genetic and physiological methods are combined to analyze the mechanisms of the neuron-recruitment and neuron-elimination. The knowledge of the new-neuron recruitment into the functioning neuronal circuit should be of critical importance in the remedy of neurological diseases that accompany neuron loss.

(3) Molecular and cellular mechanisms for the axonal recognition of specific target neurons and for the formation of specific neuronal circuits

During development, growing axons, including the axons from olfactory sensory neurons, can find their specific target neurons and form functional neuronal circuits. Recent studies on olfactory sensory neurons demonstrate a remarkable relationship between the selection of a single odorant receptor gene among a repertoire of 1000 and the address of the axon-projection target-glomeruli in the olfactory bulb. Such knowledge on the detailed address of the axon targets is currently available only in the olfactory system. We apply proteomics approaches to the olfactory axon address system to elucidate molecular mechanisms for the olfactory axon guidance to the target glomeruli and for the formation of specific synaptic connections with target neurons in the olfactory bulb.

(4) Cellular and molecular mechanisms for the contact-mediated interactions between neurons and immune cells in physiological and pathological conditions

Immune cells rarely meet central neurons in physiological conditions because of the blood brain barrier. However, a massive infiltration of immune cells into the brain occurs under many pathological conditions, suggesting that immune cells may directly interact with central neurons. To understand the nature and consequence of the direct interaction between immune cells and neurons, we are examining the change in neuronal morphology and physiological state of primary cultured neurons following the co-culture with immune cells. In addition, we are currently focusing on telencephalon-neuron specific membrane protein, telencephalin (TLCN), which bind to LFA-1 integrin expressed by leukocytes. Since the soluble form of TLCN can be detected in the serum and cerebrospinal fluid of patients of several neurological diseases, we are developing a sensitive ELISA assay system to detect the soluble TLCN for monitoring the possible neuronal damage in the telencephalic regions of the patient brain.

Future Prospects

We plan to further pursue the current research projects that are described above.

Topic (1): In addition to the current studies, we will start a new project aiming at the elucidation of neuronal substrate for the olfactory memory. In the future study an emphasis will be placed on the search for the behaviorally-relevant olfactory sensory maps (e.g., food-odor maps) at higher olfactory centers including the olfactory cortex and amygdala of the

rodent brain. An emphasis will be placed also on the search for the neuronal mechanisms associated with odorant-induced emotional changes. These studies will hopefully provide scientific basis for the therapeutic use of odorants and for the treatment of diseases in the olfactory nervous system.

Topic (2): We pursue to understand molecular and cellular mechanisms how the newly-generated neurons are integrated into the existing neuronal circuit and how the damaged-neurons are removed from the circuit without damaging the function of the neuronal circuit in the adult brain.

Topic (3): Based on our initial proteomic approaches, we now have the list of candidate molecules that might be involved in the olfactory axon target recognition. Systematic functional assays will be performed to pin-down the molecular complexes that are responsible for the proper recognition of the olfactory axon targets. The knowledge of the molecular mechanism for target recognition in the olfactory system will be of prime importance in understanding the molecular mechanism for the formation of the neuronal circuits in whole brain regions.

Topic (4): Cellular and molecular analysis of the contact-mediated interaction between immune cells and neurons will be further pursued using the co-culture assay system. In addition, we will start a systematic analysis of immune cell-mediated effects in the brain of the TLCN-deficient mice. Collaborative research with clinical laboratories will be pursued to establish the methods for detecting and measuring serum TLCN for the diagnosis of possible damage of telencephalic neurons in the brain.

Research Grants

1. Grant-in-Aid for Creative Scientific Research (JSPS) (2001-2005, ~65 millions yen / year)
2. Grant-in-Aid for Scientific Research on Priority Areas (B) (MEXT) (1999-2002, ~9.6 millions yen / year)
3. Research Grant from the Human Frontier Science Program (1999-2001, 59000 USD / year)
4. Grant-in-Aid for Exploratory Research (MEXT) (2001-2002, 1.3 millions yen / year)
5. Research Grant from Mitsubishi Foundation (2000, 7.5 millions yen)

Select Publications (1992~2002)

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Department of Neurophysiology

Outline and Research Objectives

Our laboratory was founded in 1953 as a Department of Brain Physiology in Institute for Brain Research, University of Tokyo Faculty of Medicine, and in 1996 integrated into University of Tokyo Graduate School of Medicine. We have been teaching Neurophysiology to undergraduate students in Medical School, and Master and PhD course students. As for research, using patch clamp techniques in combination with molecular techniques, we aim at elucidating cellular and molecular mechanisms underlying synaptic transmission and modulation. We are particularly interested in dynamic changes at CNS synapses during postnatal development.

Synapses in the CNS play pivotal roles in neuronal integration and plasticity. During ontogeny CNS synapses undergo protein reformations, thereby establishing mature and differentiated synaptic functions. Synapses also undergo changes in response to electrical and chemical stimuli. Various proteins in the presynaptic and postsynaptic cells are involved in such changes, and it is important to determine the role of these proteins in synaptic functions and modulations. To this end, we combine electrophysiological, molecular and morphological techniques to synapses visually identified in the CNS slices. In the rodent auditory brainstem there is a giant excitatory synapse called the calyx of Held. At this synapse it is possible to make simultaneous whole-cell recordings from a presynaptic terminal and a postsynaptic target cell (Fig. 1). Furthermore, various molecules can be loaded into a nerve terminal via whole-cell pipette perfusion during the recording (Fig.2). The large structure of this synapse also allows us to identify presynaptic proteins immunocytochemically, and even to follow translocation of presynaptic molecules upon stimulation. With these approaches in combination with genetic manipulations and protein overexpressions, we aim at addressing the molecular basis underlying synaptic functions.

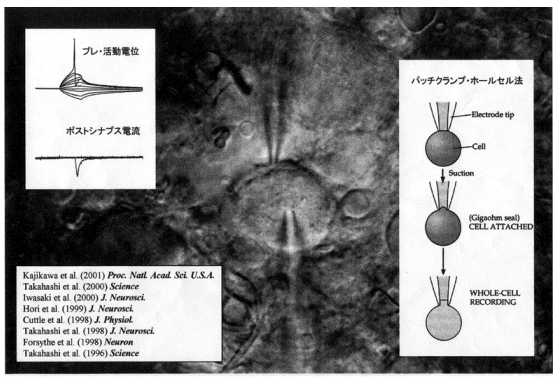

Fig. 1 Simultaneous pre- and postsynaptic whole cell recordings at the calyx of Held synapse. Inset records on the left show a presynaptic action potential and excitatory postsynaptic currents (EPSC). Righthand inset illustrates the procedure of patch-clamp whole-cell recording method.

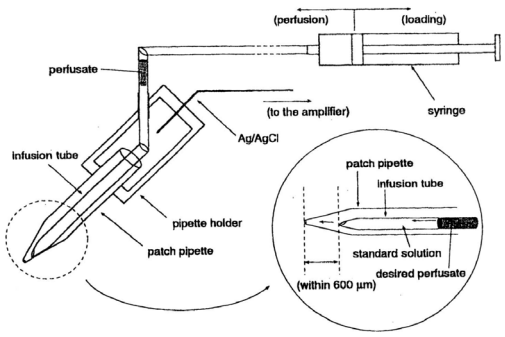

Fig. 2 Illustration of pipette perfusion system. A tube pulled from Eppendorf yellow tip is inserted into the patch pipette with its tip about 600 mm from the tip of the patch pipette.

Faculties and Students

Professor and Chair	Tomoyuki Takahashi MD, PhD (appointed since 1993)
Lecturer	Tetsuhiro Tsujimoto MD, PhD
Associate	1
Postdoctral Fellow	2
Graduate Students	9
Technical Assistant	1
Secretary	2

Past Research and Major Accomplishments

1. In simultaneous pre- and postsynaptic recordings at the calyx of Held synapse, we identified the target of presynaptic inhibition via metabotropic glutamate receptors and $GABA_B$ receptors as being the voltage-dependent Ca^{2+} channel (Takahashi et al., Science 1996, J Neurosci,1998), and that βγ subunits of the trimeric G protein Go mediate the presynaptic inhibition (Kajikawa et al, 2001 PNAS).

2. We identified that phorbol ester stimulates exocytotic machinery downstream of Ca^{2+} influx thereby causing synaptic facilitation. By loading inhibitory peptides into the nerve terminal, we also clarified that both εPKC (Fig. 4a) and the Munc13-Doc2α interaction (Fig. 4b) underlie the phorbol ester-induced facilitatory mechanism (Hori et al., J Neurosci 1998; Saitoh et al, 2002).

3. We found that Ca^{2+} channel types mediating CNS synaptic transmission switch during development (Iwasaki et al, J Neurosci 2000).

4. By loading G protein-related compounds into the calyx of Held nerve terminal, we clarified that the main role of presynaptic G proteins is to accelerate the replenishment of synaptic vesicles depleted after massive release (Takahashi et al. Science 2000).

5. We have demonstrated that the quantal analysis established at the neuromuscular junction is applicable to the CNS synapse (Sahara & Takahashi, J Physiol 2001).

6. We found that NMDA receptors are unfavorable for the reliability of fast synaptic transmission, and that their developmental decline regulated by auditory activity at the calyx of Held synapse differentiates the synapse into the high-fidelity one (Futai et al., J Neurosci 2001)

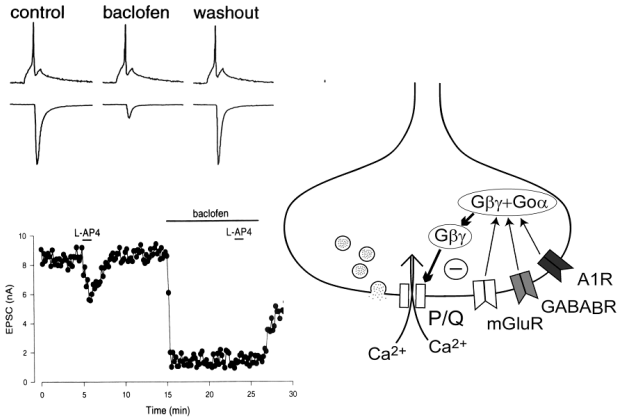

Fig. 3 Presynaptic inhibitory effect of baclofen occludes that of L-AP4, suggesting that $GABA_B$ and metabotropic glutamate receptors share a common intracellular mechanism for presynaptic inhibition. Righthand illustration summarizes our current conclusion. Adenosine A1 receptors also share the same mechanism (Kimura and Takahashi, unpublished observeation).

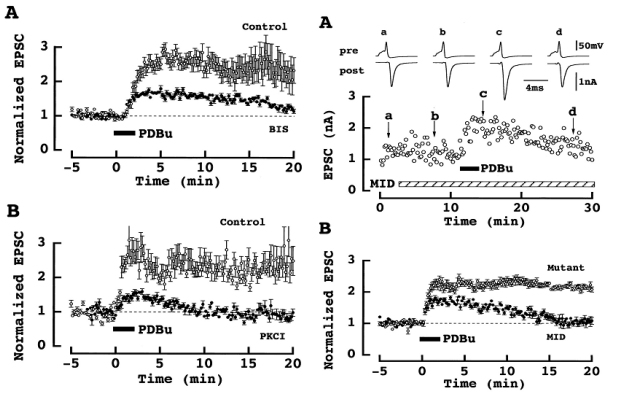

Fig. 4 *Left column*, Phorbol etser-induced EPSC potentiation is attenuated by the PKC inhibitor BIS (A) or PKC inhibtitory peptide directly loaded into the calyceal terminal (B). *Right column*, The Mid peptide, which interferes with the Doc2α-Munc13-1 interaction attenuated the phorbol etser-induced EPSC potentiation.

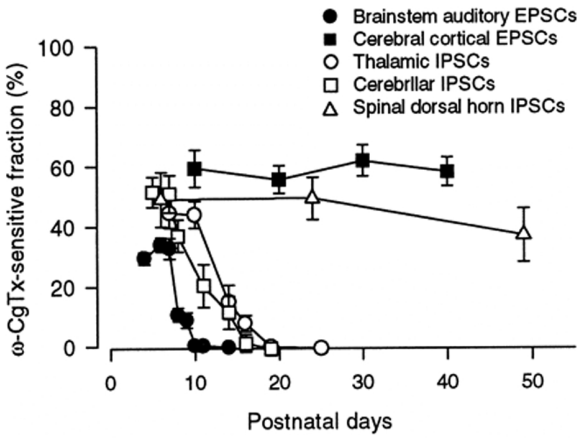

Fig. 5 Synaptic currents mediated by presynaptic N-type Ca^{2+} channels at 5 different central synapses. At 3 synapses, contribution of N-type Ca^{2+} channels to synaptic transmission decline and disappear with postnatal development, whereas at other 2 synapses it remained unchanged throughout development.

7. We found that presynaptic Ca^{2+} currents undergo facilitation by repetitive stimulation (Forsythe et al, 1998 Neuron) via acceleration of their gating kinetics (Cuttle et al., 1998 J Physiol) and that this

facilitation is mediated by the Ca^{2+} binding protein NCS-1 (Tsujimoto et al, 2002 Science).

8. We found that cytoplasmic glutamate concentration in the nerve terminal directly affects the vesicular content of glutamate and that postsynaptic AMPA and NMDA receptors are not saturated by a single packet of vesicular transmitter (Ishikawa et al, 2002 Neuron).

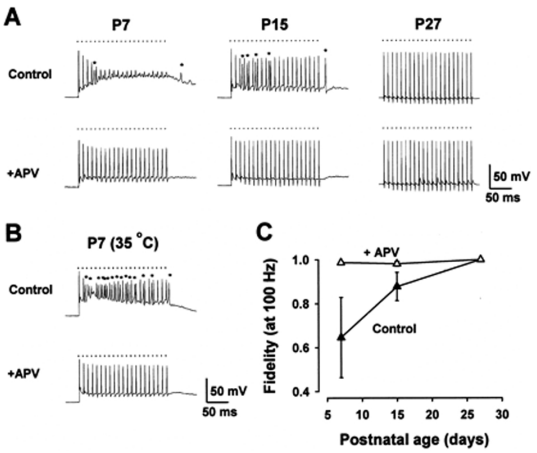

Fig. 6 Postsynaptic action potentials in response to presynaptic stimulation at 100 Hz at the developing calyx of Held synapse in mice. As mice mature fidelity of transmission increases, whereas blocking NMDA receptors using APV increases the fidelity at all ages.

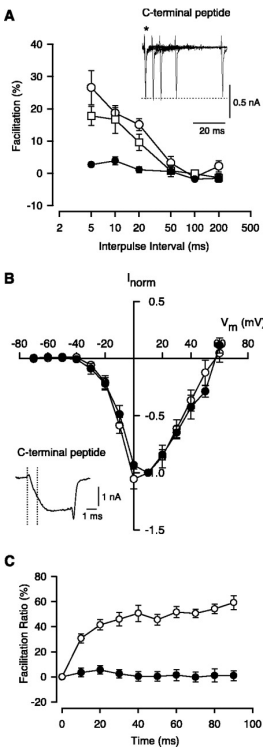

Fig. 7 The NCS-1 C-terminal peptide, when loaded into the calyx of Held presynaptic terminal, blocked the activity-dependent facilitations of presynaptic P/Q-type Ca^{2+} currents in a paired pulse protocol (A) and in a tetanic stimulation (100 Hz) protocol (C). The peptide had no effect on the current voltage relationship of presynaptic Ca^{2+} currents.

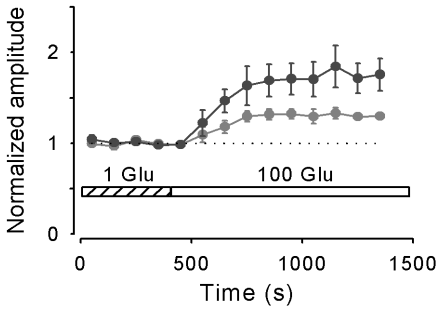

Fig. 8 Switching the L-glutamate concentration in the presynaptic whole-cell pipette from 1 mM to 100 mM markedly potentiated both quantal (blue) and evoked (red) EPSCs at the calyx of Held synapse at physiological temperature.

Current Research

1. Identification of presynaptic potassium channels involved in regulation of transmitter release.
2. Clarification on the developmental changes in the quantal synaptic responses.
3. Mechanism and roles of presynaptic adenosine receptors.
4. Developmental changes in the size of presynaptic Ca^{2+} domain.
5. Identification in the target protein downstream of εPKC.
6. Clarification in the different properties between presynaptic N-type and P/Q-type Ca^{2+} channels.

Future Prospects

1. To elucidate the minimal essential molecules required for short-term and long-term synaptic plasticity.
2. To clarify the molecular basis underlying the developmental speeding in the excitatory synaptic currents.
3. To clarify the presynaptic factor(s) determining the release probability.

Research Grants (in the past 5 years)

1996-2000

Research for the Future Program by the Japan Society of Promotion of Sciences "Molecular mechanisms of central synaptic modulation underlying the memory formation" ¥ 317,092,000

2001-2002

Grant-in-Aid for Specially promoted Research from the Ministry of Education, Culture, Sports, Science and Technology "Cellular and molecular mechanisms underlying postnatal development and differentiation of central synapses" ¥216,000,000

Select Publications

1. Ishikawa T, Sahara, Y, Takahashi T. A single packet of transmitter does not saturate postsynaptic glutamate receptors. Neuron 34, 613-621, 2002.
2. Tsujimoto T, Jeromin A, Saitoh N, Roder JC and Takahashi T Neuronal calcium sensor 1 and activity-dependent facilitation of P/Q-type calcium currents at presynaptic nerve terminals. Science 275,2276-2279, 2002.
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Department of Cellular and Molecular Pharmacology

Outline and Research Objectives

Our department was founded in 1885 as the first pharmacology department in Japan. It has had a strong research background in the field of calcium (Ca^{2+}) signalling since Professor Emeritus Setsuro Eabashi discovered in 1950's the regulatory role of intracellular Ca^{2+} concentration in muscle contraction. Since then the field of Ca^{2+} signalling research has expanded extensively: the Ca^{2+} signal is now known as a molecular switch in a vast array of important cell functions including muscle contraction, exocytosis, cell proliferation, immune responses and regulation of synaptic functions. The present research group led by Professor Iino is interested in the general principle of the Ca^{2+} signalling mechanism and is particularly interested in Ca^{2+} signalling in neurons and muscle cells at present. We have recently expanded the scope of our research to various signaling molecules upstream and downstream of Ca^{2+} signals. A notable feature of our department is that we have had an assembly of staff members with diverse backgrounds, e.g., cell physiology, molecular biology and neurobiology. We believe that the collaboration of these people will facilitate the elucidation of the medical and biological significance of Ca^{2+} signalling and related subjects.

Faculties and Students

Professor	Masamitsu Iino, M.D., Ph.D. (From April 1995)
Associate Professor	Kenzo Hirose, M.D., Ph.D.
Associate	2
Postdoctoral Fellows	3
Graduate Students	11
Research Students	3
Laboratory Staff	3

Past Research and Major Accomplishments

Our research focuses on the regulation of Ca^{2+} signals. In particular, we have made important contributions to clarifying intracellular Ca^{2+} release mechanisms. Our research also includes signaling molecules upstream and downstream of Ca^{2+} signals. Through these works, we have clarified feedback mechanisms that are essential to the generation of spatiotemporal patterns of Ca^{2+} signals.

1) Functional properties of IP_3R.

The receptor-mediated activation of phospholipase C results in the production of inositol 1,4,5-trisphosphate (IP_3), which then releases Ca^{2+} from the intracellular Ca^{2+} store. This Ca^{2+} mobilization is a central mechanism in the generation of Ca^{2+} signals for the regulation of various cell functions. We showed for the first time that the Ca^{2+} release via IP_3R is activated by submicromolar concentrations of Ca^{2+} (Fig. 1). We also discovered an inhibitory role of Ca^{2+} at higher concentrations. Thus, IP_3R activity is biphasically dependent on cytosolic Ca^{2+} concentration with the maximum level of activation obtained at 0.3 μM (*J. Gen. Physiol.*, 1990). We also showed that the effects of Ca^{2+} on IP_3R activity have no notable delay in experiments using both caged IP_3 and caged Ca^{2+} (*Nature*, 1992). These results suggest the presence of a positive feedback loop in IP_3R-mediated Ca^{2+} release. Such a positive feedback loop is expected to have a major effect on Ca^{2+} release kinetics via IP_3R (*Nature*, 1994b). This notion, which is very popular in the field of Ca^{2+} signalling, now has a strong molecular basis as shown below.

We then studied the structure-function relationship of IP_3R. IP_3R consists of three subtypes (IP_3R1, IP_3R2 and IP_3R3), that are encoded by different genes and are expressed in a tissue- and development-specific manner. In collaboration with Prof. Kurosaki of Kansai Medical University, we genetically engineered DT40 B cells to express only one IP_3R subtype and

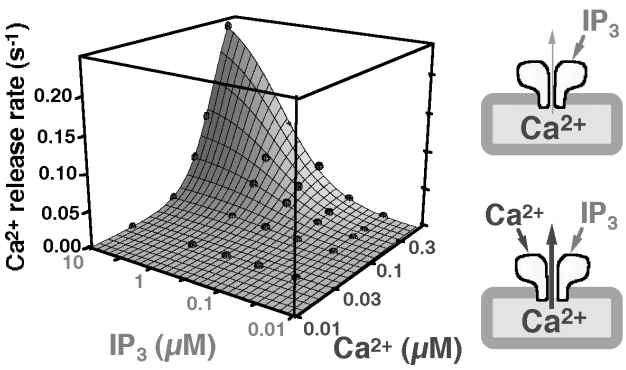

Figure 1. IP_3 and Ca^{2+} dependence of IP_3R activity. Both IP_3 and Ca^{2+} are required in the activation of IP_3R.

showed that each IP_3R subtype has distinct properties (*EMBO J.*, 1999). For example, the order of IP_3 sensitivity is $IP_3R2 > IP_3R1 > I\ P_3R3$. Furthermore, we succeeded in mapping the Ca^{2+} sensor region of IP_3R1 at glutamate 2100 (Fig. 2) (*EMBO J.*, 2001). Substitution of this amino acid by aspartate (E2100D) resulted in a 10-fold decrease in the Ca^{2+} sensitivity of IP_3R1. When we expressed the E2100D mutant IP_3R in DT40 cells, the rates of increase of Ca^{2+} concentration and Ca^{2+} oscillations during an agonist-induced response were significantly reduced compared with cells expressing wild-type IP_3R1 (Fig. 3). These results demonstrate that the Ca^{2+}-mediated feedback regulation of IP_3R activity is extremely important for the generation of spatiotemporal patterns of Ca^{2+} signals (Fig. 4).

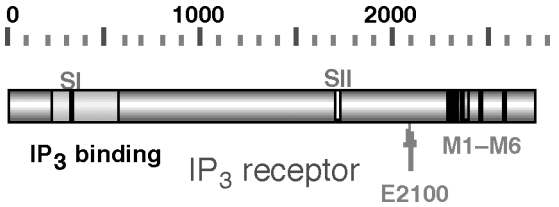

Figure 2. Ca^{2+} sensor of IP_3R. Glutamate (E) at position 2100 functions as the Ca^{2+} sensor of IP_3R.

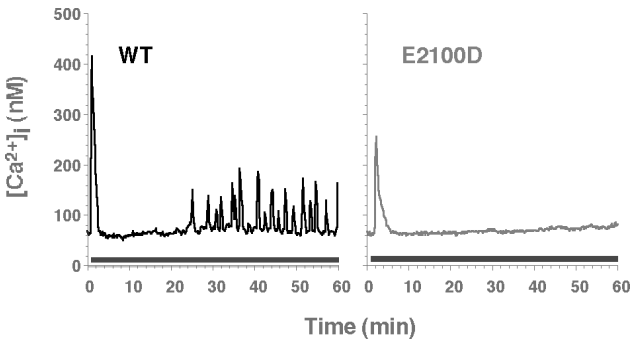

Figure 3. Ca^{2+} signalling in cells expressing mutant (E2100D) IP_3R with reduced Ca^{2+} sensitivity. BCR-mediated Ca^{2+} oscillation was abolished in cells expressing the mutant IP_3R.

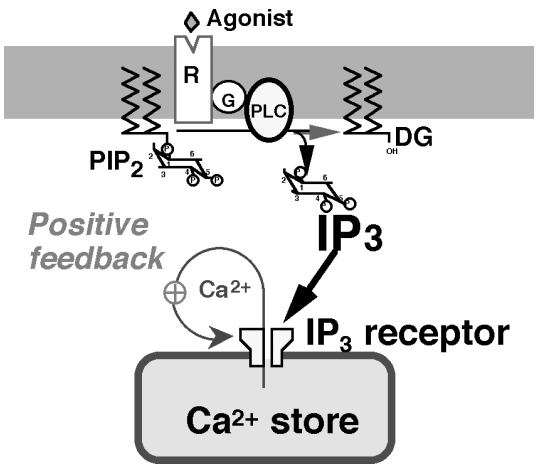

Figure 4. Positive feedback regulation of IP3R via the Ca^{2+} sensor. Ca^{2+}-sensor-mediated positive feedback regulation of Ca^{2+} release via IP3R is essential for the spatio-temporal pattern generation of agonist-stimulated Ca^{2+} release.

2) Ca^{2+} imaging using intact tissues.

In order to fully understand the physiological functions of Ca^{2+}-mediated cell signalling mechanisms, it is important to study Ca^{2+} signalling in cells that communicate with each other within intact tissues. With this notion in mind, we performed Ca^{2+} imaging experiments using intact vascular tissues. This method successfully revealed that vascular smooth muscle cells respond to sympathetic nerve stimuli with Ca^{2+} waves and oscillations (*EMBO J.*, 1994). To our surprise, resting arterial smooth muscle cells were not quiescent in terms of Ca^{2+} signals. We discovered spontaneous Ca^{2+} transients in unstimulated vascular smooth muscle cells (*J. Physiol.*, 1999). These Ca^{2+} transients are called "Ca^{2+} ripples" because their peak amplitudes were much smaller than those of sympathetic nerve-stimulated Ca^{2+} responses. Ca^{2+} ripples contribute to some extent to the resting tonus of the vascular wall. Other physiological functions await further clarification.

We have now extended the application of the tissue imaging method to intestinal tissues, and have for the first time succeeded in simultaneous imaging of Ca^{2+} signals in smooth muscle cells and interstitial cells of Cajal, which are the putative intestinal pacemaker cells (*J. Physiol.*, 2002). This method may help us elucidate the rhythm-making mechanism in intestinal tissues.

3) Excitation-contraction coupling and malignant hyperthermia.

In skeletal muscle cells depolarization of the plasma membrane induces Ca^{2+} release via the Ca^{2+} release channel (ryanodine receptor, RyR) on the adjacent sarcoplasmic reticulum. This process is called excitation-contraction (EC) coupling and has been studied using various methods. We have made several important contributions to clarifying the molecular basis of E-C coupling. There are three subtypes of RyR (RyR1, RyR2 and RyR3) encoded by different genes. First, we showed that RyR1 is critically important for EC coupling (*Nature*, 1994a). Second, we showed that RyR1 but not RyR2 or RyR3 supports the skeletal muscle EC coupling (*EMBO J.*, 1996). Third, a stretch of about 100 amino acids (D2 region), which is ~1,300 amino acids removed from the N terminus of RyR1, is important for EC coupling (*J. Biol. Chem.*, 1997). These results provided a framework for the molecular elucidation of EC coupling.

In relation to this subject, we have identified one of the genetic causes of malignant hyperthermia, a complication of general anesthesia with a life-threatening fever, resulting from an abnormality in the skeletal muscle calcium release channel, RyR1. We

identified a point mutation (L4838V) that is responsible for the gain-of-function mutation of RyR1 in malignant hyperthermia patients with marked clinical symptoms (*Jpn. J. Pharmacol.*, 2002).

We also discovered junctophilin, a protein that is responsible for the junction formation between the plasma membrane and the sarcoplasmic reticulum membrane (*Mol. Cell*, 2000).

4) IP_3 imaging and discovery of a new IP_3 signalling mechanism.

We have succeeded in visualizing the changes in intracellular IP_3 concentration using the translocation of the GFP-tagged PH domain of PLC-δ1 (GFP-PHD) (*Science*, 1999). Using this method, we showed for the first time the dynamic changes in the intracellular IP_3 concentration (IP_3 oscillations and waves) (Fig. 5). These results show that the IP_3 dynamics is an important factor for the generation of dynamic spatiotemporal patterns of Ca^{2+} signals. Furthermore, we applied this method to measuring the changes in IP_3 concentration in cerebellar Purkinje cells and found a novel Ca^{2+}-mediated IP_3 signalling pathway that leads to IP_3 production following climbing fiber inputs into Purkinje cells (*Neuron*, 2001) (Fig. 6).

Current Research

Based on the results of our studies of Ca^{2+} signalling, we recognize the importance of the spatiotemporal distribution of signalling molecules in defining cell signals. Thus, global Ca^{2+} waves and oscillations in smooth muscle cells result in *contraction* of the cells, while localized, transient rises at subplasmalemmal regions (Ca^{2+} sparks) result in *relaxation* of the cells. We, therefore, believe that it is extremely important to visualize the spatiotemporal distribution of signalling molecules within intact cells. To that end, we are currently engaged in the development of indicators of important signalling molecules (see below). Two types of excitable cells, neurons of the central nervous system and smooth muscle cells, are our current target cells. In particular, neurons have distinct cell polarity, therefore the spatiotemporal distribution of signalling molecules should be of great importance for the functions of neurons.

1) Development of novel signal indicators.

We are currently involved in the development of new genetically coded indicators of various important cell signals including nitric oxide (NO), protein phosphorylation and a Ca^{2+}-dependent transcription factor. Some of the indicators are now being expressed in cells for analysis, and others are now close to application to cells.

2) Ca^{2+} signalling in Purkinje cells and synaptic plasticity.

Ca^{2+} and IP_3 dynamics in Purkinje cells are now studied in Purkinje cells in conjunction with the molecular basis of synaptic plasticity. We use both confocal and two-photon excitation microscopies to observe signals in individual dendrites.

3) Molecular approaches to the study of IP_3R-mediated Ca^{2+} signalling.

We have identified the Ca^{2+} sensor region of IP_3R and showed that a mutation at this site resulted in the inhibition of Ca^{2+} signals. We are introducing this mutation to various cells to inhibit Ca^{2+} signals. Through this approach it will be possible to clarify the roles of Ca^{2+} signals in various cells.

Future Prospects

1) Elucidation of the relationship between Ca^{2+} signalling and cell functions.

Intracellular Ca^{2+} signals exhibit extremely dynamic changes both temporally and spatially. Such property allows the Ca^{2+} signal to be an extremely versatile switch regulating diverse cell functions; from transmitter release in neurons to cell proliferation, and from muscle contraction to apoptosis. The role of Ca^{2+} signals in skeletal muscle contraction has been thoroughly clarified. However, there are still many cell functions in which Ca^{2+} signals are thought to play important regulatory roles, but their mechanisms remain elusive. We would like to elucidate the relationship between the Ca^{2+} signalling mechanism and the regulation of cell functions in all such frontiers.

2) Visualization and analysis of molecular events at synapses during synaptic plasticity.

Among many cell functions, we are particularly interested in those of neurons, which have such a unique cell polarity and provide an excellent platform for spatiotemporal cell signalling. We are interested in cell signals upstream and downstream of the Ca^{2+} signals. Combining new signal indicators and imaging methods, we wish to visualize the spatiotemporal distribution of cell signals in order to elucidate molecular events at the synapses during synaptic plasticity that underlies learning and memory.

Research Grants

1. Core Research for Evolutional Science and Technology (CREST) from Japan Science and Technology Cooperation "Calcium Signalling Research with Advancement of Imaging and

Molecular Genetic Methods" (Years 1996-2000) ¥676,312,000.

2. Grant-in-Aid for Scientific Research (Specially Promoted Research) from the Minisitry of Education, Science, Sports and Culture. "Visualization of Intracellular Signal Flow" (Years 2000-2004) ¥430,000,000.

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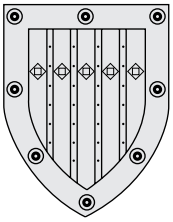

Department of Molecular Neurobiology

Outline and Research Objective

We have been investigating the molecular mechanism of brain functions. Current research activities are focused on the glutamate receptor (GluR) and learning and memory. We elucidated the molecular diversity of the *N*-methyl-D-aspartate (NMDA)-type GluR and discovered the novel δ subfamily of GluR by molecular cloning. Roles of these GluRs in brain functions have been studied by gene targeting.

Faculties and Students

Professor and Chair	Masayoshi Mishina, Ph.D (1994~)
Lecturer	Hisashi Mori, Ph.D
Associate	2 (Naoto Matsuda, M.D and Tomonori Takeuchi, Ph.D)
Postdoctoral Fellow	2
Graduate Student	11
Research Student	2
Secretary	2

Past Research and Major Accomplishments

The NMDA subtype of GluR is unique in functional properties among many neurotransmitter receptors and ion channels mediating neural signaling in the brain. The NMDA receptor channel is gated both by ligands and by voltage, and is highly permeable to Ca^{2+}. These characteristics of the NMDA receptor directly relate to its important physiological roles in synaptic plasticity as a molecular coincidence detector. Some forms of long-term potentiation (LTP) and long-term depression (LTD), which are thought to underlie learning and memory, are critically dependent on the NMDA receptor channel.

Molecular characterization of the NMDA receptor

We elucidated the molecular diversity of the NMDA receptor by molecular cloning. Highly active NMDA receptor channel was formed *in vitro* by coexpression of two members of GluR subunit families, that is, the GluRε (NR2) and GluRζ (NR1). There are four members in the ε subunit family, whereas only one member is known in the GluRζ subunit family except for the splice variants. All of the NMDA receptor channel subunits possess asparagine in segment M2. Replacement by glutamine of the asparagine in segment M2 of the GluRε2 and GluRζ1 strongly reduced the sensitivity to Mg^{2+} block of the NMDA receptor channel. Since there is strong evidence that Mg^{2+} produces a voltage-dependent block of the channel by binding a site deep within the ionophore, these results are consistent with the view that segment M2 constitutes the ion channel pore of the NMDA receptor channel.

At the embryonic stages, the GluRε2 (NR2B) subunit mRNA is expressed in the entire brain, and the GluRε4 (NR2D) subunit mRNA in the diencephalon and the brainstem. After birth, the GluRε1 (NR2A) subunit mRNA appears in the entire brain, and the GluRε3 (NR2C) subunit mRNA mainly in the cerebellum. The expression of the GluRε2 subunit mRNA becomes restricted to the forebrain, and that of the GluRε4 subunit mRNA is strongly reduced. The GluRζ1 subunit mRNA is found ubiquitously in the brain during development. The four GluRε subunits are also distinct in functional properties and regulation. Thus, multiple GluRε subunits are major determinants of the NMDA receptor channel diversity, and the molecular compositions and functional properties of NMDA receptor channels are different depending on the brain regions and developmental stages. These findings raise an important question whether the molecular diversity underlies the various physiological roles of the NMDA receptor channel.

Physiological roles of multiple NMDA receptor subtypes

To examine the functional roles *in vivo* of the diverse NMDA receptor subtypes, we generated mutant mice defective in respective GluRε subunits by gene targeting. Disruption of the GluRε1 gene results in reduction of hippocampal LTP and impairment of Morris water maze learning. In GluRε1 mutant mice, thresholds for both hippocampal LTP and contextual learning increased. The ablation of the GluRε2 subunit also impaired synaptic plasticity in the hippocampus. The reduction of GluRε1 and GluRε2 affected the plasticity of the hippocampal CA3 region in a synapse-specific manner. These results indicate that the NMDA receptor GluRε1 and GluRε2 subtypes play a key role in synaptic plasticity, learning and memory.

GluRε2 mutant mice died shortly after birth and failed to form the whisker-related neural pattern (bar-

relettes) in the brainstem trigeminal complex. In contrast, the barrelette formation was normal in GluRε4 mutant mice. These results show the involvement of the GluRε2 subunit in the refinement of the synapse formation of periphery-related neural patterns in the mammalian brain. Heterozygous mutant mice with reduced GluRε2 subunit of the NMDA receptor showed strongly enhanced startle responses to acoustic stimuli. On the other hand, heterozygous and homozygous mutation of the other NMDA receptor GluRε subunits exerted little or only small effects on acoustic startle responses. Thus, the NMDA receptor GluRε2 plays a role in the regulation of the startle reflex. GluRε4 mutant mice exhibited reduced spontaneous activity, while GluRε3 mutant mice showed little obvious deficit. GluRε1 and GluRε4 differentially contributed to pain modulation.

Discovery and functional roles of GluRδ2 in the cerebellum

The wealth of knowledge on the neural circuits in the cerebellum makes the cerebellum an ideal system to study the molecular mechanism of brain function. By molecular cloning, we found a novel GluR subfamily named GluRδ. GluRδ2 was selectively localized in cerebellar Purkinje cells. Furthermore, its intracellular localization was restricted to the parallel fiber-Purkinje cell synapses. The carboxyl terminus of GluRδ2 interacted with delphilin containing a single PDZ domain, formin homology (FH) domains and a coiled-coil structure. Analyses of GluRδ2 mutant mice revealed that the GluRδ2 subunit was essential in motor coordination and cerebellar LTD and in refinement and maintenance of Purkinje cell synapses.

We investigated eyeblink conditioning in GluRδ2 mutant mice to elucidate its cerebellar cortical neural mechanism, with reference to the temporal relationship of conditioned and unconditioned stimuli. In the delay paradigm, in which a tone (CS) overlapped temporally with a periorbital shock (US), GluRδ2 mutant mice exhibited a severe impairment in learning. However, in the trace paradigm in which a stimulus-free trace interval up to 500 ms intervened between the CS and US, GluRδ2 mutant mice learned as successfully as the wild-type mice even with 0 ms-trace interval. We then examined the delay and trace eyeblink conditioning in NMDA receptor GluRε1 mutant mice. In delay conditioning, GluRε1 mutant mice attained a normal level of the conditioned response (CR), although acquisition was a little slower than in wild-type mice. In contrast, GluRε1 mutant mice exhibited severe impairment of the attained level of the CR and disturbed temporal pattern of CR expression in trace conditioning with a longer trace interval of 500 ms. These results suggest that neural substrates underlying eyeblink conditioning are different depending on the temporal overlap of the conditioned and unconditioned stimuli.

Current Research

These studies show that GluRs play key roles in memory acquisition and neural pattern formation. The memory signaling in the adult brain may share the common molecular mechanism with the activity-dependent synapse refinement during neural development. To further investigate the molecular basis of higher brain function, we are developing the conditional gene targeting in the C57BL/6 mouse genetic background and the molecular genetics of zebrafish.

Conditional gene targeting in C57BL/6 genetic background

We developed an efficient homologous recombination system using ES cells derived from C57BL/6 strain. For a cell type-specific and temporal regulation of gene targeting in the brain, we have generated mouse lines that express Cre recombinase-progesterone receptor fusion (CrePR) gene specifically cerebellar granule cells, cerebellar Purkinje cells, hippocampal CA3 pyramidal neurons and striatal spiny neurons. Crossing of target mice first with FLP mice to remove the *neo* selection marker gene and then with Cre mice has yielded mutant mice lacking GluR genes in specific neurons.

Molecular genetics of zebrafish

Elucidation of how the neural network is formed and modulated is essential to understand how the brain is functioning. The retinotectal projection and olfactory systems in transparent zebrafish are suitable to analyze synapse formation *in vivo*. We are developing a novel strategy that visualizes and manipulates developing neurons *in vivo*.

Future Prospects

Combination of molecular genetic approaches in mice and zebrafish will facilitate our understanding of the mechanism of higher brain function at the molecular, cellular and neural network levels.

Research Grants

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