Department of Basic Medical Sciences
Division of Neuronal Network

Our major research interest is the molecular mechanisms of higher brain functions in mammals such as emotion, and learning and memory. We are especially focusing on the roles of functional molecules localized in synapses, for instance, neurotransmitter receptors, signal transduction molecules and adhesion molecules, in neuronal information processing. We are examining receptor functions, synaptic transmission and plasticity, and their roles in whole animals with electrophysiological, biochemical, molecular genetic and behavioral approaches.

1. NMDA receptor phosphorylation and synaptic plasticity

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In the hippocampus, excitatory synaptic transmission is regulated dynamically depending on the pattern of synaptic activation: high-frequency activation induces long-lasting enhancement of synaptic efficacy referred to as long-term potentiation (LTP), and prolonged lower-frequency activation causes long-term depression (LTD) of synaptic transmission. Excitatory synaptic transmission is mediated by glutamate receptors and the N-methyl-D-aspartate (NMDA) receptor, one of the glutamate receptor subtypes, plays crucial roles in LTP and LTD induction.

Tyrosine phosphorylation of NMDA receptors by Src-family tyrosine kinases such as Fyn is implicated in synaptic plasticity. We identified Fyn-mediated phosphorylation sites on the GluRε2 (NR2B) subunit of NMDA receptors. Seven out of 25 tyrosine residues in the C-terminal cytoplasmic region of GluRε2 were phosphorylated by Fyn *in vitro*. Of these seven residues, Tyr1252, Tyr1336, and Tyr1472 in GluRε2 were phosphorylated in human embryonic kidney fibroblasts when co-expressed with active Fyn, and Tyr1472 was the major phosphorylation site in this system. We then generated rabbit polyclonal antibodies specific to Tyr1472-phosphorylated GluRε2, and showed that Tyr1472 of GluRε2 was indeed phosphorylated in murine brain using the antibodies. Importantly, Tyr1472 phosphorylation was greatly reduced in *fyn*-mutant mice. Moreover, Tyr1472 phosphorylation grew evident when mice reached the age when hippocampal LTP started to be observed and its magnitude became larger. Finally, Tyr1472 phosphorylation was significantly enhanced after the induction of LTP in the hippocampal CA1 region. These data suggest that Tyr1472 phosphorylation of GluRε2 is important for synaptic plasticity.

2. Adhesion molecules and synaptic plasticity

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Adhesion molecules play critical roles in synaptic transmission and plasticity and we have examined functions of several kinds of adhesion molecules and their anchoring proteins. Telencephalin (TLCN) is a cell adhesion molecule selectively expressed in the telencephalon of the mammalian brain. The mutant mice lacking TLCN had no detectable abnormalities

in their neural development and synaptic structures. Ablation of TLCN increased LTP and its saturation level in the CA1 region of the hippocampus. The TLCN mutation selectively enhanced the performance of the radial maze and water-finding tasks, learning tasks with appetitive reinforcers, but not the contextual fear conditioning and Morris water maze tasks with aversive stimuli for conditioning. Furthermore, the TLCN mutant mice showed an increase of prepulse inhibition of the acoustic startle response. These results suggest that TLCN is a determinant of the dynamic range of synaptic plasticity and plays roles in reward-motivated learning and memory and sensorimotor gating.

3. Intracellular calcium regulation and synaptic plasticity

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The concentration of intracellular calcium is controlled by many kinds of molecules including ryanodine receptors (RyRs) and inositol trisphosphate receptors. The precise function of RyRs in synaptic transmission is unknown, while three of their subtypes are expressed in the brain. We examined the role of RyRs in excitatory synaptic transmission in hippocampal slices, using type 3 RyR (RyR3)-deficient mice. The α-amino-3-hydroxy-5-methyl-4-isoxozolepropionic acid (AMPA) receptor-mediated basal synaptic responses in the CA1 region of mutant mice were smaller than those of wild-type mice, while there was no difference in NMDA receptor-mediated responses, suggesting selective postsynaptic modification of AMPA receptors by RyR3. The expression of synaptic AMPA receptors examined by Western blotting or immunohistochemistry was indistinguishable, suggesting that the smaller AMPA synaptic responses in mutant mice were not due to the reduced number of synaptic AMPA receptors. Although the initial potentiation was similar, LTP was smaller in mutant mice. There were no differences in presynaptic electrophysiological properties. We conclude that RyR3 regulates the properties of AMPA receptors postsynaptically.

4. Regulation of synaptic glutamate concentrations and synaptic plasticity

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At the excitatory synapse, the neurotransmitter glutamate released from the presynaptic terminal is subject to the reuptake by glial or neuronal glutamate transporters. Although glutamate transporters maintain low extracellular levels of glutamate in the nervous system, little is known about their roles in synaptic plasticity. Here, using knockout mice lacking GLT-1 that is the most abundant glial subtype of glutamate transporters, we showed that LTP induced by tetanic stimulation in mutant mice was impaired in the hippocampal CA1 region. When tetanic stimulation was applied in the presence of low concentrations of an NMDA receptor antagonist, the impairment was overcome. Consistent with these results, the increased glutamate in the synaptic cleft of mutant mice preferentially activated NMDA receptors. Furthermore, analyses of mutant mice revealed that the magnitude of NMDA receptor-dependent transient synaptic potentiation during low-frequency stimulation depended on the concentration of glutamate in the synaptic cleft. These findings suggest that GLT-1 plays critical roles in LTP induction, as well as in short-term potentiation, through regulation of extracellular levels of glutamate, which enables appropriate NMDA receptor activation.

5. Analysis of muscarinic acetylcholine receptor functions using knockout mice

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We are investigating the biological function of muscarinic acetylcholine receptors (mAChRs) using mutant mice lacking corresponding genes. These mice have been established by Matsui et al. in the Laboratory of Biomedical Genetics, Graduate School of Pharmaceutical Sciences, University of Tokyo (Prof. Makoto Mark Taketo's Lab.).

The mAChR belongs to a group of seven transmembrane-spanning receptors and is distributed widely in both central and peripheral nervous systems. Five distinct genes for mAChRs were cloned in rats and humans. Now, it is generally accepted that five classes of affinity (M_1, M_2, M_3, M_4 and M_5) defined by various muscarinic ligands are attributable to these gene products, respectively. Previously, Matsui et al. have cloned the all five genes for mouse mAChRs (*Chrm1*, *Chrm2*, *Chrm3*, *Chrm4*, and *Chrm5*), and determined the chromosomal locations of these genes.

Elucidation of the subtype-specific functions of mAChRs has been a matter of considerable interest because they are suitable targets for various therapeutic drugs. However, due to poor subtype selectivity of the available ligands, pharmacological attempts to elucidate the role of each subtype remain inconclusive. As an alternative and more powerful

strategy, we are studying the receptor function using our knockout mice for M_1, M_2, M_3, M_4, and M_5. In order to minimize the contaminating effects caused by different genetic backgrounds, we are carefully backcrossing these animals to C57BL/6J and DBA/2J strains.

We have previously reported that the M_3 knockout mice are retarded in post-weaning growth and devoid of pilocarpine-induced salivation. The mice also showed partial mydriasis, and male-selective urinary retention. We are now comparing the phenotype of M_3 knockout mice with that of M_2 knockout mice, because M_2 and M_3 are the two major subtypes in smooth muscle organs. Furthermore, we have established a double knockout mouse line lacking both M_2 and M_3 and analyzing their phenotypes.

In addition, mAChRs are supposed to be important in several aspects of brain functions. These include learning and memory, drug addiction, sleep and respiratory control, and striatal function. We are investigating the role of each subtype in these functions, employing molecular biology, electrophysiology, and behavioral experiments.

Our mice are now regarded as invaluable resources in the research community and we are organizing many collaborative programs (both domestic and international) as well.

6. Role of MAP kinase signaling in synaptic plasticity

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Several lines of evidence including ours have suggested that the mitogen-activated protein kinase (MAPK) signaling cascade plays a regulatory role in the induction of LTP. We therefore examined the change in synaptic transmission and plasticity in genetically manipulated mice that carry no SynGAP, a GTPase-activating protein known to interact with PSD-95 and negatively regulate MAPK signaling. The mutant mice showed a reduced level of LTP at all examined protocols and showed deficits in a hippocampus-dependent spetial learning test, which can be overcome by excess training. The molecular mechanisms underlying this LTP and learning impairment is still unclear, and the studies to identify the altered MAPK pathways in the mutant mice are now in progress.

7. Modulatory neurotransmitters and synaptic plasticity

Ayako M. Watabe, Hideki Miwa, Thomas J. O'Dell[9], and Toshiya Manabe

Several signaling mechanisms that are crucial for the induction of LTP by theta frequency (5 Hz) trains of synaptic stimulation are altered in aged animals. Thus, to determine whether the induction of LTP by theta frequency stimulation is particularly sensitive to changes in synaptic function that occur in aged animals, we compared the effects of three different trains of synaptic stimulation pulses delivered at 5 Hz (theta pulse stimulation, TPS) on synaptic strength in the hippocampal CA1 region of aged and young mice. In addition, we investigated whether the modulation of TPS-induced LTP by β-adrenergic and cholinergic receptor activation showed deficits with aging. Our results indicated that TPS-induced LTP was not diminished in the aged hippocampus but showed pronounced dependence on L-type calcium channels that was not seen in slices from young animals. In addition, we observed that the enhancement of TPS-induced LTP by co-activation of β-adrenergic and cholinergic receptors was significantly reduced in slices obtained from aged animals. Since TPS-induced LTP was not altered in aged mice, our results suggest that deficits in modulatory pathways that regulate activity-dependent forms of synaptic plasticity may contribute to memory impairments in older animals. The molecular and biochemical mechanisms underlying this alteration in aged animals are currently under investigation.

8. Mechanisms of bidirectional synaptic modification

Ayako M. Watabe, Hideki Miwa, Noriko Kumazawa, Thomas J. O'Dell[9], and Toshiya Manabe

a. Characterization of mGluR-dependent synaptic plasticity

Activity-dependent modification of synaptic strength plays a key role in neural development and some forms of neuronal plasticity. While much focus has been on the LTP mechanisms, not much is known for the molecular mechanisms of LTD, long-lasting suppression of synaptic strength. Recently, it has been reported that activation of the metabotropic glutamate receptor (mGluR) with the group I mGluR agonist (R,S)-3,5-dihydroxyphenylglycine (DHPG) induces LTD in the CA1 region of the hippocampus. We investigated potential roles of pre- and postsynaptic processes in the DHPG-induced LTD. DHPG-induced LTD was completely blocked when GDP-βS was delivered into postsynaptic cells, strongly suggesting that DHPG depresses synaptic transmission through a postsynaptic, G protein-mediated signaling pathway. On the other hand, the effect of DHPG was strongly modulated by experimental manipulations that altered presynaptic calcium influx. Also, enhancing calcium influx by

prolonging action potential duration with bath applications of the potassium channel blocker 4-AP strongly reduced the effect of DHPG. Furthermore, while inhibiting both pre- and postsynaptic potassium channels with bath-applied 4-AP blocked the effects of DHPG, inhibition of postsynaptic potassium channels alone with intracellular cesium and TEA had no effect on the ability of DHPG to inhibit synaptic transmission. These results suggest that activation of postsynaptic mGluRs suppresses transmission at excitatory synapses onto CA1 pyramidal cells through presynaptic effects on transmitter release. Further physiological roles of mGluRs in synaptic transmission and activity-dependent modification of synaptic transmission are currently in progress.

b. Molecular mechanisms of metaplasticity

While certain patterns of synaptic stimulation can change synaptic strength, the degree and/or direction of the synaptic modification itself can strongly depend on the previous history of the synaptic stimulation. This effect of the stimulus history onto the plasticity, or plasticity of plasticity (*metaplasticity*), has been implicated from the theoretical point of view in neuronal network development, but its physiological and biochemical mechanisms are still unclear. To elucidate molecular and cellular mechanisms underlying metaplasticity, possible involvement of mGluRs and NMDA receptors and their modulation such as phosphorylation and dephosphorylation are currently investigated.

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