Department of Basic Medical Sciences

Division of Neuronal Network
神経ネットワーク分野

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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 the whole animal with electrophysiological, biochemical, molecular genetic and behavioral approaches.

1. NMDA receptor phosphorylation and synaptic plasticity

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In a variety of brain regions, excitatory synaptic transmission is regulated dynamically depending on the pattern of synaptic activation: high-frequency activation induces long-lasting enhancement of the 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 and Tyr 1472 was the major phosphorylation site. We generated mice with a knockin mutation of the Tyr-1472 site to phenylalanine (Y1472F) and show that Tyr-1472 phosphorylation is essential for functions of the amygdala. The knockin mice show impaired fear-related learning and reduced amygdaloid LTP. NMDA receptor-mediated calcium/calmodulin-dependent protein kinase II (CaMKII) signaling is impaired in YF/YF mice. Electron-microscopic analyses reveal that the Y1472F mutant of the NR2B subunit shows improper localization at synapses. We thus identify Tyr-1472 phosphorylation as a key mediator of synaptic plasticity and fear-related learning in the amygdala.

2. Inhibitory modulation of synaptic plasticity is stronger in the dentate gyrus than in the CA1 region of the hippocampus.

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Long-term potentiation (LTP) is a phenomenon that the efficacy of synaptic transmission is enhanced after high-frequency activation of the synapse. It was first discovered in the hippocampus, and it has been widely accepted as a cellular basis of certain forms of memory. In the medial perforant pathway-dentate gyrus granule cell synapse and in the CA3-CA1 pyramidal cell synapse, LTP is induced by a similar mechanism (postsynaptic N-methyl-D-aspartate receptor dependent), while several reports suggested that the modulation of LTP by γ-aminobutyric acid type A ($GABA_A$) receptor-mediated inhibitory inputs is stronger in the medial perforant pathway-dentate gyrus granule cell synapse. To explore the underlying molecular mechanism that makes the difference between the two regions, we compared LTP in the presence of the $GABA_A$ receptor antagonist picrotoxin with LTP in its absence in the CA1 region and in the dentate gyrus using acute slices of the rat hippocampus. We then compared the inhibitory monosynaptic responses with excitatory monosynaptic responses, and also compared their summation during an LTP-inducing stimulus between the two regions. Our results suggest that the stronger inhibitory modulation of LTP in the dentate gyrus may be due to the balance biased towards inhibition between the summated inhibitory and excitatory postsynaptic currents during conditioning in the dentate gyrus. Besides these examinations of synaptic inhibitory inputs, several reports suggested that continuous activation of extrasynaptic $GABA_A$ receptors by ambient GABA is different in several aspects between the two regions, which could also contribute to the finding about LTP modulation as well. For example, it is reported that continuous activation of extrasynaptic $GABA_A$ receptors is mediated by the receptor with different subunit compositions between the two regions, which may result in different properties of the inhibition. Thus, we are currently examining whether this kind of inhibition is associated with the stronger inhibitory modulation of LTP in the dentate gyrus using the whole-cell patch-clamp technique.

3. Functional properties of the NMDA receptor in the lateral amygdala: a comparison with those in the hippocampal CA1 region

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The amygdala is a crucial brain structure for the acquisition and expression of fear memory. The N-methyl-D-aspartate (NMDA)-type glutamate receptor channel, composed of the NR1 (GluRζ) and NR2 (GluRε) subunits, plays a key role in synaptic plasticity in the central nervous system. NR2 subunits (NR2A-NR2D) are differentially expressed, depending on developmental stages and brain regions, but their functional roles in the amygdala are still largely unknown. Here, we have investigated the properties of synaptic NMDA receptors in the lateral nucleus of the amygdala (LA), comparing them with those in the hippocampal CA1 region. We find that the biophysical properties of NMDA receptors and NR2A/NR2B ratio in the LA are distinct from those in the CA1 region and that the NR2B subunit contributes to synaptic transmission and LTP induction to a greater extent in LA than in the CA1 region. Our data suggest that these properties of NMDA receptors in the LA are responsible for unique properties of amygdaloid synaptic function and plasticity.

4. 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 (mAChR KO mice). These mice have been established by Matsui *et al.* at Laboratory of Biomedical Genetics, Graduate School of Pharmaceutical Sciences, University of Tokyo (Prof. Makoto M. Taketo Lab). The mAChRs (M_1, M_2, M_3, M_4 and M_5) belong to a group of seven transmembrane-spanning receptors and are distributed widely in both the central and peripheral nervous systems. Elucidation of the subtype-specific functions of mAChRs has been a matter of considerable interest, especially because they are suitable targets for pharmacological therapeutics. However, because of poor subtype-selectivity of the available ligands, pharmacological approaches to discriminate their roles remain inconclusive.

The use of mAChR KO mice is an alternative strategy to achieve complete subtype specificity. In order to minimize the concomitant effects reflecting the possible difference in the genetic background, we have backcrossed most of these mutant lines to two representative inbred stra-

ins, C57BL/6J and DBA/2J, for more than 10 generations. Various compound mutant mice (M_1/M_2, M_1/M_3, M_1/M_4, M_1/M_5, M_2/M_3, M_2/M_4, and M_3/M_5) are also available.

We are investigating the significance of each subtype, employing molecular biology, electrophysiology, and behavioral experiments. The achievement of this year includes elucidation of mAChR functions in smooth muscle contraction /relaxation, salivary secretion, regulation of GABAergic neuron activity in the dorsal horn, and regulation of the endocannabinoid signaling in the striatum (see the publication list for details).

5. Dynamics of the actin cytoskeleton in dendritic spines: roles in morphological regulation and synaptic plasticity

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Dendritic spines of pyramidal cells in the mature brain receive excitatory inputs. Each spine provides a postsynaptic biochemical compartment. Since Santiago Ramón y Cajal discovered dendritic spines of neurons more than 100 years ago, it has been a long-lasting question whether shapes of spines are related to their function. Recent advanced techniques of imaging GFP-tagged proteins reveal that spine shapes are unexpectedly dynamic, responding to glutamate stimulation. The actin cytoskeleton predominates in spines, and regulates their morphological plasticity and the anchoring of certain postsynaptic molecules. Numerous studies suggest that actin remodeling is a key to understand the molecular mechanism underlying activity-dependent morphological changes. Stability and mechanical property of actin filaments are generally regulated by their side-binding proteins. This project aims to elucidate a role of reorganization of the spinous actin cytoskeleton in synaptic functions.

Drebrin, one of the actin side-binding proteins, is highly enriched in dendritic spines of the mature brain. Using immunoelectron microscopy and a newly-developed antibody against drebrin A, we have shown that drebrin A, a neuron-specific isoform of drebrin, localizes in sites of prospective excitatory synapses in the immature brain. We have also found that 20 % of dendritic spines contain no drebrin. Since Alzheimer's disease shows major loss of drebrin in the dendritic spine and since down-regulation of the drebrin-A isoform caused by antisense oligonucleotides induces cognitive deficits, we hypothesize that the drebrin content in a dendritic spine is closely related to its synaptic function. It has been immunohistochemically shown that down-regulation of the drebrin-A isoform caused by antisense oligonucleotides in developing cultured hippocampal neurons prevents spine formation and PSD-95 accumulation in dendritic spines. We are now interested in a role of drebrin in trafficking of glutamate receptors during synaptogenesis.

We have reported that intense stimulation with glutamate induces the translocation of drebrin from dendritic spines to their parent dendrites. The translocation of drebrin might be a cause of actin reorganization associated with synaptic activity. Further immunohistochemical and DiI-labeling studies on the effects of glutamate on spine shapes are now in progress. The ionic mechanisms underlying the drebrin translocation have also been examined using glutamate receptor antagonists and Ca^{2+} channel blockers. We are now investigating the ATPase-dependent mechanism of the drebrin translocation. We have just started to examine the effects of ATPase inhibition on synaptic plasticity such as LTP at excitatory synapses in the CA1 region of the rat hippocampus.

6. Spatial and temporal patterns of the signal propagation in hippocampal neuronal circuits: gating mechanisms in the dentate gyrus and the CA2 region in the hippocampal network

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The lamellar hypothesis in the hippocampus is based on physiological data showing that stimulation of the entorhinal cortex activates only a limited number of CA1 cells arranged in a direction along the alvear fibers of the hippocampus. A simple tri-synaptic circuit (DG-CA 3-CA1), which is based on classical anatomical observations with Golgi staining, is consistent with the lamellar hypothesis. However, this hypothesis has been criticized because recent anatomical work has revealed that there is wider distribution of axons along the longitudinal axis of the hippocampus than expected in the simple tri-synaptic concept, and that there are much richer connections among hippocampal subfields (DG, CA3, CA2, and CA1). The discrepancy between results of physiological and anatomical experiments may be due to the inhibitory mechanisms that suppress signal propagation be-

yond lamellar organization. To examine whether such an inhibitory mechanism is present between lamellae in the rat hippocampus, hippocampal slices were prepared transversely (at a right angle to the long axis), and obliquely (along the alvear fibers). The mossy fiber stimulation evoked population spikes of CA1 neurons in the oblique slices, but not in the transverse slices. These data are consistent with the trisynaptic circuit classically proposed in the lamellar hypothesis. We found that an adenosine A_1 receptor antagonist, 8-cyclopentyltheophylline (8-CPT), produced population spikes in CA 1 neurons in the transverse slices. These data indicate that endogenous activity of adenosine A_1 receptors is involved in the inhibition of signal propagation from the CA3 to CA1 region beyond lamellar organization. We have started to analyze spatial and temporal patterns of the signal propagation from the CA3 to CA1 region evoked by the mossy fiber stimulation in oblique and transverse slices using a newly developed low-noise CMOS sensor. We have immunohistochemically shown that adenosine A_1 receptors are highly expressed in the CA2 region. Optical recordings using a voltage-sensitive dye would enclose whether CA2 neurons are activated by the application of 8-CPT and whether the activation of CA2 neurons is the source of the CA1 activity.

We are currently interested in a role of the supramammillary nucleus (SuM) of the hypothalamic nucleus in the hippocampal function, because the SuM neurons send dense fibers directly to the dentate gyrus and the CA2 region. We have previously shown that intrasupramammillary injection of the $GABA_A$ receptor agonist muscimol prevents the generation of seizure discharges in the rat hippocampus of a kainic acid-induced epileptic model. Our findings suggest that inputs from the SuM to the hippocampus gate the signal flow from the entorhinal cortex to the hippocampus. We have started a new project on signal propagation from the entorhinal cortex to the dentate gyrus using the horizontal slice preparation in which the connection between the two brain regions is preserved.

We hypothesize that the SuM controls the hippocampal memory function. We have tried to trace the fiber tracts from the SuM to the hippocampus with a tracer injection. Since the CA2 region is in the position which controls longitudinal signal propagation in the hippocampal formation, it is important to assess when and how CA2 neurons are activated in vivo. We have analyzed the number of Fos-immunopositive neurons (FN) in the supramammillary nucleus (SuM) and the hippocampus of the rats that had been placed in an open field. Further, we have analyzed effects of SuM lesions on the increase of FN in the CA2 region. The CA2 region was identified by the absence of the mossy fibers. We are preparing papers on these results.

7. Regulation of adenosine A_1 receptor expression

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Pertussis toxin functionally uncouples adenosine A_1 receptor (A_1AR) from its effectors. We hypothesized that this loss in the receptor coupling could lead to de novo A_1AR synthesis by the cell in a futile attempt to re-establish normal receptor function. To test this hypothesis, we used hamster ductus deferens tumor (DDT1 MF-2) cells, a cell culture model for studying A_1AR, and showed that pertussis toxin (100 ng/ml) produced a time-dependent loss in A_1AR-G_i interaction and abolished A_1AR activation of extracellular signal regulated kinase (ERK)1/2. Interestingly, pertussis toxin increased the expression of A_1AR, as measured by real time PCR, immunocytochemistry and [^{3}H]-cyclopentyl-1,3-dipropylxanthine (DPCPX) binding, suggesting a compensatory response to G_i protein inactivation. Inhibition of NF-κB attenuated the increase in A_1AR induced by pertussis toxin. We conclude that pertussis toxin promotes de novo A_1 AR synthesis by activating NF-κB through an ADP ribosylation-independent mechanism involving intracellular Ca^{2+} release and PKC activation.

Further, we evaluated the role of NO in the regulation of A_1AR expression, a G protein-coupled receptor involved in cytoprotection in the central nervous system, as the expression of the A_1AR is regulated by oxidative stress. Administration of the NO donor, S-nitrosylpenicillamine (SNAP), to pheochromocytoma 12 (PC 12) cells increased A_1AR protein in a time- and dose-dependent manner. The response to SNAP was attenuated by the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5- tetramethylimidazoline -1-oxyl-3 oxide (C-PTIO), and by the inhibition of nuclear factor-jB (NF-jB), implicating this transcription factor in the regulatory process. In addition, SNAP also increased the degradation of Inhibitory jB-a (IjB-a), a marker of NF-jB activation. Furthermore, the induction of inducible nitric oxide synthase (iNOS) by lipopolysaccharide increased A_1AR in PC12 cells and in mice,

whereas the inhibition of NOS activity suppressed this response. We conclude that NO, via the activation of NF-jB, serves as an endogenous regulator of A_1AR, and speculate that the induction of the A_1AR could counteract the cytotoxicity of NO.

8. GABAergic interneurons facilitate mossy fiber excitability in the developing hippocampus.

Michiko Nakamura, Yuko Sekino, and Toshiya Manabe

Profound activity-dependent synaptic facilitation at hippocampal mossy fiber synapses is a unique and functionally important property. Although presynaptic ionotropic receptors, such as kainate receptors, contribute partially to the facilitation in the hippocampus, the precise mechanisms of presynaptic regulation by endogenous neurotransmitters remain unclear. In this study, we report that axonal $GABA_A$ receptors on mossy fibers are involved in the activity-dependent facilitation during development. In immature mouse hippocampal slices, short-train stimulation (5 pulses at 25 Hz) caused frequency -dependent facilitation of not only postsynaptic responses but also presynaptic fiber volleys that represent presynaptic activities. This fiber volley facilitation was inhibited by selective $GABA_A$ receptor antagonists, or by enkephalin that selectively suppresses excitability of interneurons. Furthermore, we directly demonstrated that this facilitation resulted from depolarization of mossy fibers in imaging experiments using a voltage-sensitive dye. This increased mossy fiber excitability caused by depolarizing action of GABA gradually decreased with development and eventually disappeared at around postnatal day 30. These results suggested that GABA released from interneurons acted on axonal $GABA_A$ receptors on mossy fibers and contributed at least partially to the activity- and age-dependent facilitation in the hippocampus.

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