DEPARTMENT OF REGULATION BIOLOGY

Chairman: Masaharu Noda

The Department is composed of two regular divisions and two adjunct divisions. The study of this department is focused on molecular mechanisms for the development of central nervous systems in vertebrates, and also on molecular mechanisms for the response of plants toward external environments, such as light, temperature and salinity.

DIVISION OF MOLECULAR NEUROBIOLOGY

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We have been studying the molecular and cellular mechanisms underlying the development and functioning of the vertebrate central nervous system. We are currently searching for and analyzing the functions of molecules involved in various cellular events in brain morphogenesis and brain function, such as generation of neuroblasts, migration to form the laminar structure and various nuclei, elongation and path-finding of neural processes, the formation and refinement of specific connections between neurons, and also synaptic plasticity. We have been using various techniques including molecular biology, biochemistry, immunological methods, neuroanatomy, cell and organotypic culture, embryo manipulation (gene transfer and gene targeting), and behavioral analysis.

I. Molecular mechanism of the retinotectal projection

Topographic maps are a fundamental feature of neural networks in the nervous system. Understanding the molecular mechanisms by which topographically ordered neuronal connections are established during development has long been a major challenge in developmental neurobiology. The retinotectal projection of lower vertebrates including birds has been used as a readily accessible model system. In this projection, the temporal (posterior) retina is connected to the rostral (anterior) part of the contralateral optic tectum, the nasal (anterior) retina to the caudal (posterior) tectum, and likewise the dorsal and ventral retina to the ventral and dorsal tectum, respectively. Thus, images received by the retina are precisely projected onto the tectum in a reversed manner. In 1963, Sperry proposed that topographic mapping could be guided by complementary positional labels in gradients across pre- and postsynaptic fields. Although this concept is widely accepted today, and Eph families of receptor tyrosine kinases and their ligands were recently identified as candidates for such positional labels, the molecular mechanism of retinotectal map formation remains to be elucidated.

Since 1993, we have been devoting our efforts to searching for topographic molecules which show asymmetrical distribution in the embryonic chick retina. In the first-round screening using a cDNA subtractive hybridization technique, we identified two winged-helix transcriptional regulators, CBF-1 and CBF-2, expressed in the nasal and temporal retina, respectively. Furthermore, our misexpression experiments using a retroviral vector showed that these two transcription factors determine the regional specificity of the retinal ganglion cells, namely, the directed axonal projections to the appropriate tectal targets along the anteroposterior axis. To further search for topographic molecules in the embryonic retina, we next performed a large-scale screening using a new cDNA display system called Restriction Landmark cDNA Scanning (RLCS). With the assistance of a computer image-processing software, we successfully identified 33 molecules along the nasotemporal axis and 20 molecules along the dorsoventral axis, with various asymmetrical expression patterns in the developing retina. We have elucidated the primary structures of all these cDNA clones and examined their expression patterns during development. These included many novel molecules together with the known molecules: transcription factors (CBF-2, COUP-TFII, etc.), receptor proteins (EphA3, EphB3, etc.), secretory factors, intracellular proteins, and so on.

Among them, we recently identified a novel retinoic acid (RA)-generating enzyme, RALDH-3, which is specifically expressed in the ventral region of the retina, together with a dorsal-specific enzyme RALDH-1. In chick and mouse embryos, the expression of *Raldh-3* is observed first in the surface ectoderm overlying the ventral portion of the prospective eye region earlier than that of *Raldh-1* in the dorsal retina, and then the *Raldh-3* expression shifts to the ventral retina (Fig. 1). Furthermore, we found that *Raldh-3* is a downstream target of *Pax6* which is known to be the master gene for the eye development in many species. It is well known that RA is essential for the eye development. These results suggest that RALDH-3 and RALDH-1 are the key enzymes for the dorsoventral patterning at the early stage of the retinal development.

Currently, with respect to the identified topographic molecules, we are conducting over- and misexpression experiments using viral vectors and *in ovo* electroporation to elucidate their molecular functions. We expect that our studies will lead to elucidation of the molecular mechanism underlying the retinal patterning and topographic retinotectal projection, and ultimately to uncovering the basic principles for establishing complicated

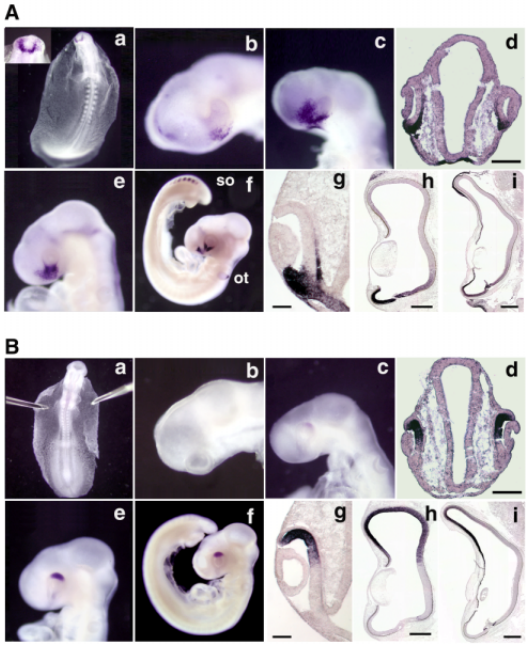

Fig.1 Expression patterns of *Raldh-3* (A) and *Raldh-1* (B) during chick development
All embryos were hybridized with DIG-labeled antisense riboprobes. Developmental stages of embryos are stage 10 (A, a), stage 11 (B, a), stage 12 (A and B, b), stage 14 (A and B, c, d), stage 16 (A and B, e), stage 20 (A and B, f, g), E6 (A and B, h), and E8 (A and B, i), respectively. The expression of *Raldh-3* is observed first in the surface ectoderm overlying the ventral portion of the prospective eye region earlier than that of *Raldh-1* in the dorsal retina. Coronal sections are oriented in dorsal up and ventral down. so, somites; ot, otic vesicle. Scale bars: 100 μm (A and B, d, g, h); 200 μm (A and B, i).

but extremely precise neural networks.

II. Functional roles of protein tyrosine phosphatase ζ

Protein tyrosine phosphorylation plays crucial roles in various aspects of brain development. The level of tyrosine phosphorylation is determined by the balance between the activities of protein tyrosine kinases and protein tyrosine phosphatases. Many types of receptor-type protein tyrosine phosphatases (RPTP) have been cloned and characterized. In 1994, we found that PTPζ/RPTPβ, a nervous system-rich RPTP, is expressed as a chondroitin sulfate proteoglycan in the brain. An RNA splice variant corresponding to the extracellular region of PTPζ is secreted as a major proteoglycan in the brain known as 6B4 proteoglycan/phosphacan. The extracellular region of PTPζ consists of a carbonic anhydrase-like domain, a fibronectin-type III-like domain and a serine-glycine-rich region, which is considered to be the chondroitin sulfate attachment region. PTPζ is expressed from the early developmental stage to the adulthood. This suggests that this molecule plays variegated roles in the brain development and brain functions.

In an attempt to reveal the signal transduction mechanism of PTPζ, we first tried to identify the ligand molecules of this receptor. We found that PTPζ binds pleiotrophin/HB-GAM and midkine, closely related heparin-binding growth factors which share many biological activities. The chondroitin sulfate portion of PTPζ is essential for the high affinity binding (Kd = ~0.25 nM) to these growth factors, and removal of chondroitin sulfate chains results in a marked decrease of binding affinity (Kd = ~13 nM). We further revealed that chondroitin sulfate interacts with Arg^{78} in Cluster I, one of the two heparin-binding sites in the C-terminal half domain of midkine. This is the first demonstration that chondroitin sulfate plays an important regulatory role in growth factor signaling.

Next, we examined the roles of pleiotrophin/midkine-PTPζ interaction in neuronal migration using the glass fiber assay and Boyden chamber cell migration assay. Pleiotrophin and midkine on the substratum stimulated migration of neurons in these assays. Polyclonal antibodies against the extracellular domain of PTPζ, 6B4 proteoglycan (a secreted extracellular form of PTPζ) and sodium vanadate (a protein tyrosine phosphatase inhibitor) added to the culture medium strongly suppressed this migration. Experiments using various midkine mutants with various affinities for PTPζ indicated that the strength of binding affinities and the neuronal migration-inducing activities are highly correlated. These results suggest that PTPζ is involved in migration as a neuronal receptor for pleiotrophin and midkine.

In order to reveal the intracellular signaling mechanism of PTPζ, we performed yeast two-hybrid screening using the intracellular region of PTPζ as bait. We found that PTPζ interacts with PSD-95/SAP90 family members, SAP102, PSD-95/SAP90 and SAP97/hDlg, which are concentrated in the central synapses mediating protein-protein interactions to form large synaptic macromolecular complexes. Here, the C-terminus of PTPζ binds to PSD-95/SAP90 proteins through the second PDZ domain. This suggests that PTPζ is involved in the regulation of synaptic function. However, PSD-95/SAP90 family members are not likely to be the substrate for PTPζ because this family members are not tyrosine-phosphorylated.

To identify the substrate molecules of PTPζ, we have recently developed the yeast substrate-trapping system. This system is based on the yeast two-hybrid system with two essential modifications: conditional expression of v-src to tyrosine-phosphorylate the prey proteins and screening using a substrate-trap mutant of PTPζ as bait (Fig. 2). Using this system, we identified GIT1/Cat-1 as a PTPζ substrate. PTPζ and GIT1/Cat-1 were colocalized in the growth cones of mossy fibers from pontine explants and in the ruffling membranes and processes of B103 neuroblastoma cells (Fig. 2). Moreover, pleiotrophin increased tyrosine phosphorylation of GIT1/Cat-1 concomitantly with its recruitment to the stimulated PTPζ in B103 cells. It is known that Cat-1 regulates Pak, a serine threonine kinase which serves as a target for the small GTP-binding proteins, Cdc42 and Rac, and is implicated in a wide range of cellular events

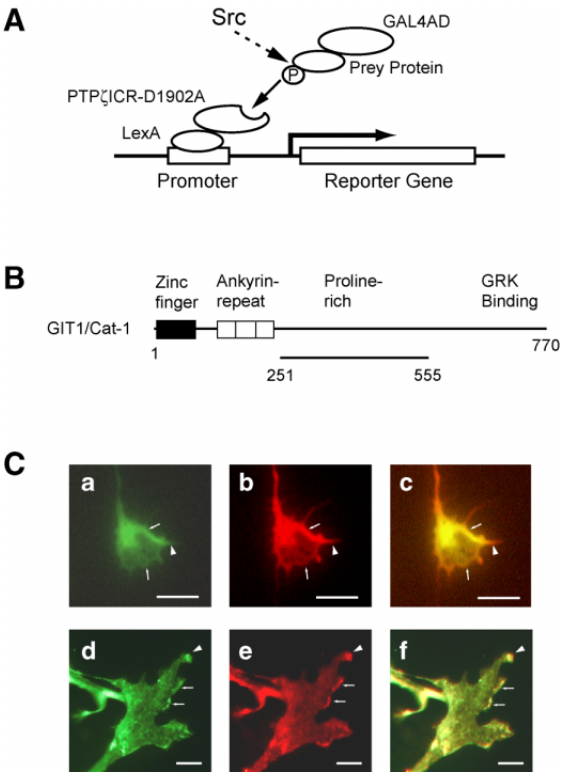

Fig. 2 The yeast substrate-trapping system and GIT1/Cat-1
(A) In the presence of 1 mM methionine when v-src is not induced, only the standard two-hybrid bindings occur. In the absence of methionine, prey proteins (substrates for PTPζ) could be phosphorylated by the induced v-src, and trapped by the bait consisting of the whole intracellular domain with an Asp1902Ala mutation (PTPζ ICR-D1902A). This complex formation leads to activation of transcription of the reporter genes, *HIS3* and *LacZ*. We performed screening in two steps and selected the colonies which showed an increase in blue color development upon induction of v-src.
(B) GIT1/Cat-1 contains a zinc-finger region, three ankyrin-repeat regions, a potential SH3-binding site and a GRK binding site. The region encoded by the clone isolated by the yeast substrate-trapping system is shown with a solid line underneath.
(C) The mossy fibers extending from pontine explants were doubly immunostained with anti-GIT1/Cat-1 (a) and anti-6B4 PG (b) antibodies. The combined image is shown in (c). The colocalization of both proteins was evident at the peripheral regions (arrows) and filopodial processes (arrowhead) of the growth cones. B103 cells were doubly immunostained with anti-GIT1/Cat-1 (d) and anti-6B4 PG (e) antibodies. The combined image is shown in (f). GIT1/Cat-1 and PTPζ were colocalized in the processes (arrowhead) and ruffling membranes (arrows) of the cells. Scale bars: 5 μm (a-c), 10 μm (d-f).

including the cell adhesion and cell morphological change. Pleiotrophin, PTPζ and GIT1/Cat-1 might regulate the neuronal migration and neurite extension by controlling the Pak signaling pathway.

To further study the physiological function of PTPζ *in* vivo, we generated PTPζ-deficient mice in which the PTPζ gene was replaced with the LacZ gene in 1997. By investigating the expression of LacZ in heterozygous mutant mice, we demonstrated that neurons as well as astrocytes express PTPζ in the central nervous system. We are currently studying the phenotype of PTPζ-deficient mice biochemically, anatomically, physiologically and ethologically, and have already found abnormalities in behavior, learning and memory, etc.

III. Physiological roles of Na_v2 ion channel

Voltage-gated sodium channels (NaChs) are responsible for generating action potentials in excitable cells and play many important physiological roles. Cloning of NaChs revealed marked conservation in the primary structures that underlies their functional similarity. Thus, all cloned NaChs had been grouped into a single gene family. However, recently, novel NaChs, human $Na_v2.1$, mouse $Na_v2.3$ and rat NaG/SCL11, were cloned from inexcitable cells such as glial cells. These molecules closely resemble each other but are divergent from the previously cloned sodium channels including the regions involved in activation, inactivation and ion selectivity. Thus, these molecules should be grouped into a new subfamily of NaChs (Na_v2). To date, Na_v2 channels have not been expressed in a functionally active form using *in vitro* expression systems, and therefore the functional properties of these NaChs are not yet clear.

To clarify the physiological function and the cells expressing Na_v2 channels *in vivo*, we generated knockout mice in which Na_v2 channel gene was replaced with the *LacZ* or *neo* gene by gene targeting. In this study, we found that mouse $Na_v2.3$ and rat NaG/SCL11 genes are species counterparts. Analysis of the targeted mice allowed us to identify Na_v2-producing cells by examining the *lacZ* expression. Besides in the lung, heart, dorsal root ganglia and Schwann cells in the peripheral nervous system, *Na_v2* was expressed in neurons and ependymal cells in restricted areas of the central nervous system, particularly in the circumventricular organs that are involved in body-fluid homeostasis (see Fig. 1A in the part of Center for Transgenic Animals and Plants). Under water-depleted conditions, the mutant mice showed markedly elevated *c-fos* expression in neurons in the subfornical organ and organum vasculosum laminae terminalis compared with wild-type animals. This suggests that these neurons are in a hyperactive state in the *Na_v2*-null mice. Moreover, the null mutants showed abnormal intakes of hypertonic saline under both water- and salt-depleted conditions (Fig. 1B, *ibid.*). These findings suggest that the Na_v2 channel plays an important role in the central sensing of the body-fluid sodium level, and in regulation of salt intake behavior. We are currently examining the electrophysiological property of the cells derived from Na_v2-null mutant mice and wild-type mice to gain insight into the channel property of Na_v2.

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