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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 (*e.g.* cDNA cloning, site-directed mutagenesis Yeast two-hybrid system), biochemistry (protein, carbohydrate), immunological methods (monoclonal-antibody production), neuroanatomy, cell and organotypic culture (immortalized cell-line production), and embryo manipulation (classical embryology, gene transfer with viral vectors, and gene targeting).

I. Molecular mechanism of retinotectal map formation

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 suggested 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. We examined whether CBF-1 and CBF-2 control the expression of EphA3, a promising candidate for a topographic guidance label in the retina, using *in ovo* electropolation. We obtained results suggesting that these two transcription factors affect the topographic expression of EphA3 indirectly.

In order to further search for topographic molecules in the embryonic retina, we performed a large-scale screening using a new cDNA display system called Restriction Landmark cDNA Scanning (RLCS). A number of molecules displaying various asymmetrical expression patterns along the nasotemporal axis or dorsoventral axis in the retina have been identified. These included already known topographic molecules such as EphA3, CBF-2, etc, expressed along the nasotemporal axis, and ephrin-B2, EphB3, etc, expressed along the dorsoventral axis. We have identified all of the cDNA clones isolated by this screening and examined their expression patterns during development. Furthermore, with respect to the topographic molecules which might have important roles in formation of the retinotectal map and/or the neural network in the retina, we conducted over- and misexpression experiments using viral vectors and *in ovo* electropolation to elucidate the molecular function. In 1999, with the assistance of a computer image-processing software (Fig. 1), we found additional ~30 and ~80 candidate cDNA spots for topographic molecules along the nasotemporal and dorsoventral axes, respectively. Currently, projects to identify them and analyze the expression pattern are underway.

We expect that our studies will lead to elucidation of the molecular mechanism underlying formation of the regional specificity in the retina, and ultimately to uncovering the basic principles for establishing complicated 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

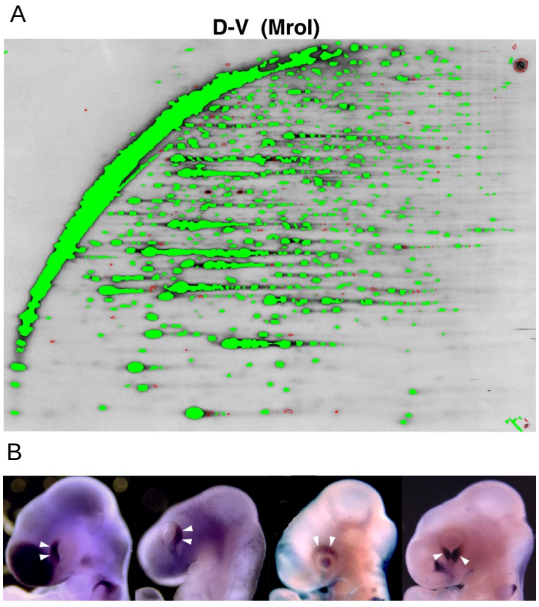

Fig. 1 Genes expressed in a region-specific fashion in the chick retina
(A) RLCS profiles for the dorsal and ventral retina. The dorsal profile (black) was overlaid with the ventral profile (green) using an image-processor. Dorsal specific clones are detectable as rare black spots among abundant green spots.
(B) Region-specific gene expression in the retina. Whole-mount in situ hybridization showed that these genes were specifically expressed in the nasal, temporal, dorsal or ventral region of the retina, respectively.

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-specific 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.

In an attempt to reveal the signal transduction mechanism of PTPζ, we tried to identify the ligand molecules of this receptor. To date, we have 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.

In the embryonic rat brain, pleiotrophin and midkine are localized along the radial glial fibers, a scaffold for neuronal migration. On the other hand, PTPζ is expressed in the migrating neurons, suggesting that the ligand-receptor relationship between these molecules plays a role in migration of neurons during brain development. Thus, 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 suggested that PTPζ is involved in migration as a neuronal receptor for pleiotrophin and midkine distributed along radial glial fibers.

Next, in order to reveal the intracellular signaling mechanism of PTPζ, we performed yeast two-hybrid screening using the intracellular region of PTPζ as bait. By screening a rat brain cDNA library, we found that PTPζ interacts with PSD-95/SAP90 family molecules, SAP102, PSD-95/SAP90 and SAP97/hDlg. These proteins are composed of three PDZ domains, a SH3 domain and a guanylate kinase-like domain, and are concentrated in the central synapses mediating protein-protein interactions to form large synaptic macromolecular complexes. Using the yeast two-hybrid binding assay, we found that the C-terminal sequence of PTPζ binds to PSD-95/SAP90 proteins through the second PDZ domain (Fig. 2). Immunohistochemical analysis revealed that PTPζ and PSD-95/SAP90 are similarly distributed in the dendrites of pyramidal neurons of the hippocampus and neocortex (Fig. 2). These results suggest that PTPζ is also involved in the regulation of synaptic function.

To 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 behaviour, circadian rhythm, LTP in the hippocampus, etc.

III. Functional roles of subfamily 2 sodium channels

Voltage-gated sodium channels (NaChs) are responsible for generating action potentials in excitable cells and play many important physiological roles. Cloning

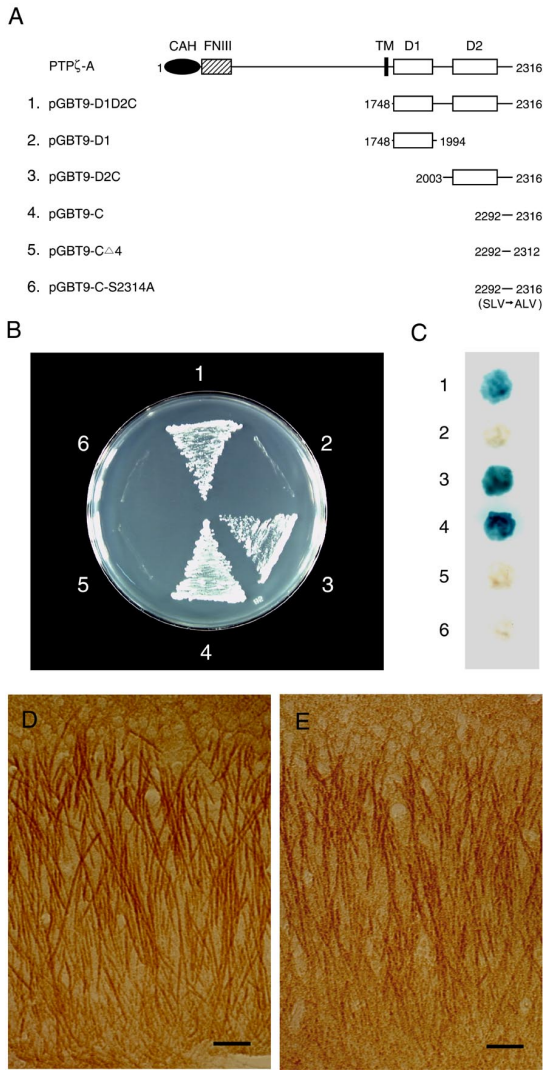

Fig. 2 Interaction of PTPζ with PSD-95/SAP90
Schematic representations of PTPζ and yeast two-hybrid constructs are shown in (A). Various bait constructs of PTPζ were tested for interaction with PSD-95/SAP90 by induction of reporter genes, *HIS3* (B) and *LacZ* (C). Numbers in (B) and (C) correspond to the construct numbers in (A). These results and the other experiments indicated that the C-terminal portion of PTPζ interacts with the second PDZ domain of PSD-95/SAP90. Immunohistochemical staining with the antibodies against PTPζ (D) and PSD-95/SAP90 (E) indicated that both proteins were present in the dendrites of hippocampal pyramidal cells. Scale bars: 40 μm.

of NaChs revealed marked conservation in primary structure that underlies their functional similarity. Thus, all NaChs cloned had been grouped into a single gene family. However, recently, novel NaChs, human Nav2.1, mouse Nav2.3/mNaG and rat SCL11/NaG, were cloned from inexcitable cells such as glial cells. These molecules closely resemble each other but are divergent from the previously cloned sodium channels even in the regions involved in activation, inactivation and ion selectivity. Thus, these molecules have been grouped into a new subfamily of NaChs (subfamily 2). To date, subfamily 2 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 cells expressing subfamily 2 channels and their physiological functions *in vivo*, we planned to generate knock-out mice deficient in the channel gene.

We successfully generated mutant mice in which the mouse subfamily 2 channel gene (*mNaG*) was replaced with the *LacZ* or *neo* gene by gene targeting. From the analysis of *LacZ* knock-in mice, we demonstrated that *mNaG* gene expression is restricted to the dorsal root ganglion and lung during the embryonic stage (see Fig. 1A in the part of Center for Transgenic Animals and Plants). During the postnatal period, in addition to these tissues, Schwann cells in the sensory afferent nerve fibers (*ibid*. Fig. 1B, C) and a subset of neurons in the central nervous system were positive for *mNaG* gene expression. We are currently examining the phenotypes of *mNaG*-null mutant mice to gain insight into the physiological functions of this channel.

Publication List:

Takahashi, M., Yamagata, M. and Noda, M. (1999) Specific expression of ezrin, a cytoskeletal-membrane linker protein, in a subset of chick retinotectal and sensory projections. *Eur. J. Neurosci.* **11**, 545-558.

Yamakawa, T., Kurosawa, N., Kadomatsu, K., Matsui, T., Itoh, K., Maeda, N., Noda, M., and Muramatsu, T. (1999) Levels of expression of pleiotrophin and protein tyrosine phosphatase ζ are decreased in human colorectal cancers. *Cancer Letter*, 135, 91-96.

Maeda, N., Ichihara-Tanaka, K., Kimura, T., Kadomatsu, K., Muramatsu, T. and Noda, M. (1999) A receptor-like protein tyrosine phosphatase PTPζ/RPTPβ binds a heparin-binding growth factor midkine: Involvement of arginine 78 of midkine in the high affinity binding to PTPζ. *J. Biol. Chem*., **274**, 12474-12479.

Revest, J.-M., Faivre-Sarrailh, C., Maeda, N., Noda, M., Schachner, M. and Rougon, G. (1999) The interaction between F3 immunoglobulin domains and protein tyrosine phosphatase ζ/β triggers bidirectional signalling between neurons and glial cells. *Eur. J. Neurosci*. **11**, 1134-1147.

Kawachi, H., Tamura, H., Watakabe, I., Shintani, T., Maeda, N. and Noda, M. (1999) Protein tyrosine phosphatase ζ/RPTPβ interacts with PSD-95/SAP90 family. *Mol. Brain Res*., **72**, 47-54.

Yamagata, M., Mai, A., Pollerberg, G.E. and Noda, M. (1999) Regulatory interrelations among topographic molecules CBF1, CBF2 and EphA3 in the developing chck retina. *Dev. Growth Differ*. **41**, 575-587.

Meng, K., Rodorigues-Peña, A., Dimitrov, T., Chen, W., Yamin, M., Noda, M. and Deuel, T.F. (2000) Pleiotrophin signals increased tyrosine phoshorylation of β-catenin through inactivation of the intrinsic catalytic activity of the receptor-type protein tyrosine phosphatase β/ζ. *Proc. Natl. Acad.Sci.* USA **97**, 2603-2608.

Shintani, T., Maeda, N. and Noda, M. (2000) Receptor-like protein tyrosine phosphatase γ (RPTPγ), but not PTPζ/RPTPβ, inhibits NGF-induced neurite outgrowth in PC12D cells. *Dev. Neurosci.*, in press.

Nishiwaki, T., Maeda, N., and Noda, M. (1999) Characterization and developmental regulation of proteoglycan-type protein tyrosine phosphatase ζ/RPTPβ isoforms. *In* Neural Development (Uyemura, K., Kawamura, K., and Yazaki, T. *eds.*) pp.291-297. Springer-Verlag Tokyo.