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We have been studying the molecular and cellular mechanisms underlying the development of the vertebrate central nervous system (CNS), mainly using mice. This research covers many developmental events including the patterning of the retina, neuronal terminal differentiation, axonal navigation, branching and targeting, synapse formation, refinement and plasticity. The scope of our interests also encompasses the mechanisms for various functions of the mature brain, including body-fluid regulation, behavior control, learning, and memory.

I. Mechanisms for neural circuit formation

Topographic maps are fundamental features of neural networks in the nervous system. We have long studied the molecular mechanisms for regional specification in the developing retina as the basis of the topographic retinotectal projection. We are now focusing our attention on the molecular mechanisms underlying axonal navigation, branching, and arborization for synapse formation, along with elimination of mistargeted axons and branches. Among the region-specific molecules in the developing retina, we have already found several molecules that induce abnormal branching or arborization when their expression was experimentally manipulated *in vivo*.

One is adenomatous polyposis coli 2 (Apc2), which is preferentially expressed in the nervous system from early developmental stages through to adulthood. The knockdown of *Apc2* in chick retinas reduced the stability of microtubules in retinal axons, and yielded abnormal behaviors of growth cones including a reduced response to ephrin-A2 and misprojection in the tectum without making clear target zones. In *Apc2*-deficient mice, robust defects in neuronal lamination were observed in the cortex, hippocampus, cerebellum, and olfactory bulb. These laminary abnormalities are a result of dysregulated neuronal migration by a cell-autonomous mechanism. Apc2 is distributed along actin fibers as well as microtubules in neurons. Our investigation suggests that Apc2 is involved in the signaling pathway from membrane receptors for extracellular guidance factors to the intracellular migration machinery. The phenotypes observed in *Apc2*-deficient mice suggest that mutations in *APC2* in humans may cause a neurodevelopmental disorder.

Another molecule is SPARC-related protein containing immunoglobulin domains 1 (SPIG1), which is a secretory protein expressed in a region-specific manner in the developing retina. The knockdown of *SPIG1* in chick retinas induces abnormal branching and arborization of retinal axons on the tectum. We are now investigating the molecular mechanism underlying the regulation of axon branching by SPIG1.

II. Development of direction-selective retinal ganglion cell subtypes

Visual information is transmitted to the brain by roughly a dozen distinct types of retinal ganglion cells (RGCs) defined by characteristic morphology, physiology, and central projections. However, because few molecular markers corresponding to individual RGC types are available, our understanding of how these parallel pathways develop is still in its infancy.

The direction of image motion is computed in four cardinal directions and coded by direction selective (DS) ganglion cells in the retina. Particularly, the ON DS ganglion cells are critical for mediating the optokinetic reflex. We generated a knock-in mouse in which SPIG1-expressing cells are labeled with GFP. We successfully visualized both upward-motion-preferring and downward-motion-preferring ON DS ganglion cells (SPIG1^{+} and SPIG1^{-} ganglion cells, respectively) by a combination of genetic labeling and conventional retrograde labeling in the medial terminal nucleus.

It is not known at which circuit location along the flow of visual information the cardinal direction selectivity first appears. In collaboration with Dr. Roska's Group, we

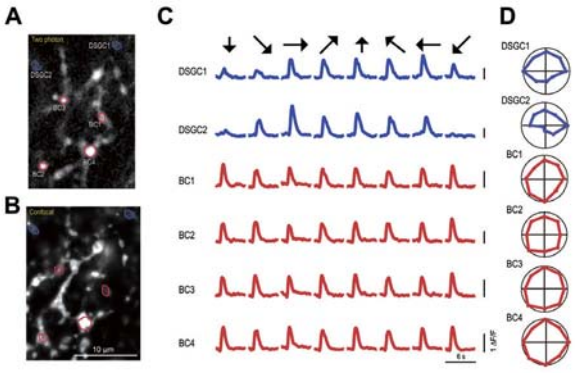


Figure 1. Concerted activity at subcellular resolution within the circuit of a single ON DS ganglion cell. **A**, Top view of two-photon image of bipolar cell axon terminal and ON DS ganglion cell dendrite labeled with GCaMP3 expressed from transsynaptic rabies virus initiated from an ON DS ganglion cell. Regions of interest are marked by colored lines. BC, bipolar cell; DSGC, ON DS ganglion cell. **B**, Confocal picture of the same region as in (A). **C**, Calcium transients in ON DS ganglion cell dendrites (blue) and bipolar cell axon terminals (red) recorded by two-photon imaging in response to stimuli moving in eight different directions. Labels to the left of traces indicate locations of the recorded compartments in (A). **D**, Polar plots of peak responses to each direction of motion.

recorded the concerted activity of the neuronal circuit elements of single ON DS ganglion cells at subcellular resolution by combining GCaMP3-functionalized transsynaptic viral tracing and two-photon imaging. While the visually evoked activity of the dendritic segments of the DS ganglion cells were direction selective, direction-selective activity was absent in the axon terminals of bipolar cells (Figure 1). Furthermore, the glutamate input to DS ganglion cells, recorded using a genetically encoded glutamate sensor, also lacked direction selectivity. Therefore, these results suggest that cardinal direction selectivity appears first at the dendrites of DS ganglion cells.

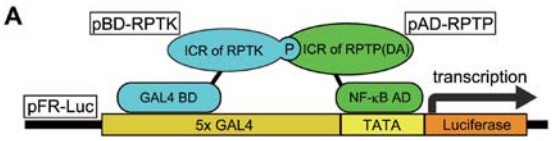
III. Physiological roles of receptor-like protein tyrosine phosphatases

Protein tyrosine phosphorylation plays crucial roles in various biological events such as cellular proliferation, differentiation, survival, migration, and metabolism. Cellular tyrosine phosphorylation levels are governed by the opposing activities of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). The physiological functions and regulatory mechanisms of receptor-like PTPs (RPTPs) are not fully elucidated. We have been making efforts to reveal the functional roles of the R3 and R5 subfamilies of RPTPs.

3-1 R3 RPTP subfamily

The human genome contains 58 and 20 genes for RPTKs and RPTPs, respectively. In some studies including ours, RPTPs have been shown to be involved in the regulation of RPTKs through dephosphorylation as substrates. However, our understanding about the roles of individual RPTPs in the regulation of RPTKs is still limited.

The R3 RPTP subfamily, which is comprised of Ptprb, Ptprh, Ptprj, and Ptpro, reportedly plays pivotal roles in the development of several tissues including the vascular and nervous systems. We examined enzyme-substrate relationships between the four R3 RPTP subfamily members and 21 RPTK members selected from 14 RPTK subfamilies by using a mammalian two-hybrid system with substrate-trapping RPTP (RPTP(DA)) mutants (Figure 2A). Among the 84 RPTP-RPTK combinations conceivable, we detected 30 positive interactions: 25 of the enzyme-substrate relationships were novel (Figure 2B). We randomly chose several RPTKs assumed to be substrates for R3 RPTPs, and validated the results of this screen by *in vitro* dephosphorylation assays, and by cell-based assays involving overexpression and knock-down experiments. Because their functional relationships were verified without exception, it is probable that the RPTKs identified as potential substrates are actually physiological substrates for the R3 RPTPs. Interestingly, some RPTKs were recognized as substrates by all R3 members, but others were recognized by only one or a few members: This study is the first to demonstrate the similarities and differences in substrate-specificities among a RPTP subfamily. The enzyme-substrate relationships identified in the present study will shed light on physiological roles of the R3 RPTP subfamily.



B

RPTPs

RPTKs	Ptpro	Ptprb	Ptprj	Ptprh
EphA4	+++	+++	++	++
EphB2	+++	+++	++	++
TYRO3	+++	+++	++	++
RET	+++	+++	+++	–
MET	++	+++	+++	–
RON	–	–	–	–
LTK	+	+++	++	–
FGFR1	+	++	–	–
ERBB2	++	+++	–	–
EGFR1	–	–	–	++
ROS	–	+++	–	–
PDGFRa	–	+	–	–
VEGFR1	–	–	–	++
VEGFR2	–	–	–	++
VEGFR3	–	–	–	–
DDR1	–	–	–	–
DDR2	–	–	–	–
ROR1	–	–	–	–
ROR2	–	–	–	–
TIE1	–	–	–	–
TRKB	–	–	–	–

Figure 2. Identification of RPTK-RPTP interactions by a mammalian two-hybrid system using substrate-trapping mutants of RPTP. **A**, The strategy to detect RPTK-RPTP interactions. COS7 cells were transfected with a pBD-RPTK (encoding a fusion protein GAL4 BD domain and intracellular region (ICR) of RPTK) and a pAD-RPTP (encoding a fusion protein NFkB AD and ICR of RPTP(DA)) expression plasmid, together with the reporter plasmid pFR-Luc. Thus, the protein interactions can be assessed with a standard luciferase assay. Luciferase activities induced are expected to correlate with the strength of interaction. **B**, Summary of specific interactions between DA mutants of RPTP and RPTKs. The experiments were carried out in triplicate. Signal intensities were classified into four groups: -, none; +, weak; ++, medium; +++, strong.

3-2 R5 RPTP subfamily

Protein-tyrosine phosphatase receptor type Z (Ptprz) is predominantly expressed in glial and neuronal cells in the central nervous system (CNS). We are now focusing our efforts on determining the roles of Ptprz signaling in the regulation of hippocampal synaptic plasticity, dopamine transporter internalization, oligodendrocyte differentiation, etc.

IV. Brain systems for body-fluid homeostasis

Sodium (Na) is a major electrolyte of extracellular fluids and the main determinant of osmolality. Na homeostasis is essential to life and Na^+ concentrations in plasma and cerebrospinal fluid (CSF) are continuously monitored to maintain a physiological level of Na^+ in body fluids. We have previously shown that Na_x, which structurally resembles voltage-gated sodium channels ($Na_v1.1$–1.9), is a concentration-sensitive Na channel.

In the brain, Na_x channels are preferentially expressed in astrocytes and ependymal cells in the sensory circumventricular organs, such as the subfornical organ (SFO) and organum vasculosum of the lamina terminalis

(OVLT), where Na_x-positive glial cells are involved in sensing an increase in $[Na^+]$ in body fluids. *Na_x*-deficient mice do not stop ingesting salt even when dehydrated, while wild-type mice avoid salt. This behavioral defect of *Na_x*-deficient mice is recovered by a site-directed transfer of the *Na_x* gene with an adenoviral vector into the SFO. Na_x thus functions as the brain's Na^+-level sensor for the homeostatic control of $[Na^+]$ in body fluids.

In our previous *in vitro* studies, Na_x showed a threshold value of ~150 mM for $[Na^+]_o$. However, $[Na^+]$ in serum and CSF is strictly controlled at 135–145 mM in mammals including humans, suggesting that the brain sensor(s) can detect an increase in $[Na^+]$ in this range to strictly maintain the physiological level. We therefore presumed that the threshold value of Na_x for $[Na^+]_o$ must be modulated *in vivo* by some mechanism.

Recently, we found that endothelin-3 (ET-3) shifts the $[Na^+]_o$ dependency of Na_x activation to the lower-concentration side in a dose-dependent manner (Figure 3A and B). This shift enables Na_x to gate even when $[Na^+]_o$ is in the physiological range. ET_BR signaling promoted activation of Na_x through protein kinase C (PKC) pathways leading to extracellular signal-regulated kinase 1/2 (ERK1/2) activation. Importantly, expression of ET-3 in the SFO was time-dependently enhanced during dehydration (Figure 3C). Moreover, BQ788, a specific blocker of ET_BR attenuated the salt-aversive behavior in wild-type mice induced by dehydration (Figure 3D). These results strongly indicate that regulated expression of ET-3 in the SFO is involved in the control of salt-intake behavior through improvement of the $[Na^+]_o$ sensitivity of Na_x *in vivo*.

Very recently, we demonstrated that Na_x was involved in the regeneration process of injured peripheral nerves by enhancing lactate release from non-myelinating Schwann cells, where Na_x was activated by ET-1 through ET_BR signaling. This finding may bring new strategies to promote peripheral nerve regeneration.

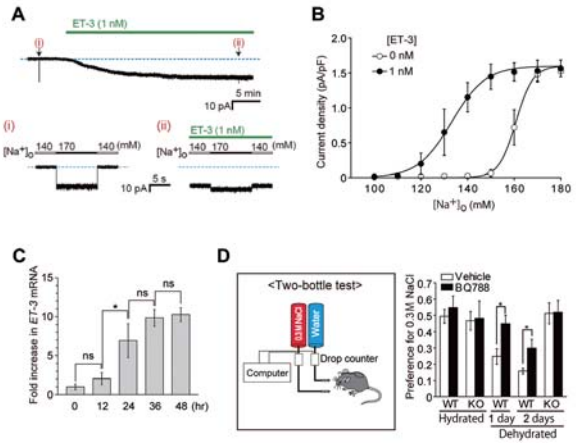


Figure 3. ET-3 expression in the subfornical organ enhances the sensitivity of Na_x to suppress salt intake. **A**, A representative whole-cell current response of a SFO cell obtained from WT mice on application of hypertonic Na^+ during ET-3 application. The transient application of hypertonic Na^+ (170 mM) was performed at the time points indicated by arrows, 5 min before (i) and 30 min after (ii) the start of the ET-3 application (1 nM). The dotted lines represent the basal level under the 140 mM $[Na^+]_o$ condition without ET-3. The currents induced by an increase in $[Na^+]_o$ (+30 mM) at the time points (i) and (ii) are magnified below. **B**, Relationships between the current density and $[Na^+]_o$ in the presence or absence of 1 nM ET-3. The current amplitudes from the basal level were measured and plotted. For comparisons, the current changes due to the shift of the E_{Na} were compensated. Data are mean ± SEM (n = 6 for each point). The $C_{1/2}$ values were determined after curves fitting (solid lines). **C**, Changes in the signal intensity of the *in situ* hybridization for ET-3 mRNA in the SFO during dehydration. Signal intensities are shown as relative values against that at 0 hr. *$p < 0.05$, two-tailed Student's t test; ns, not significant; data are mean ± SEM (n = 8 for each). **D**, Preference ratios for 0.3 M NaCl in the two-bottle test during 12 hr. Wild-type mice (WT) and *Na_x*-deficient mice (KO) received an i.c.v. infusion of BQ788 or vehicle were compared. The preference ratio was defined as the ratio of the volume of 0.3 M saline ingested to total fluid intake. *$p < 0.05$, two-tailed Student's t test; data are mean ± SEM (n = 10 for each).

Publication List

〔Original papers〕

- Ayoub, E., Hall, A., Scott, A.M., Chagnon, M.J., Miquel, G., Hallé, M., Noda, M., Bikfalvi, A., and Tremblay, M.L. (2013). Regulation of the Src kinase-associated phosphoprotein 55 homologue by the protein tyrosine phosphatase PTP-PEST in the control of cell motility. J. Biol. Chem. *288*, 25739-25748.
- Hiyama, T.Y., Yoshida, M., Matsumoto, M., Suzuki, R., Matsuda, T., Watanabe, E., and Noda, M. (2013). Endothelin-3 expression in the subfornical organ enhances the sensitivity of Na_x, the brain sodium-level sensor, to suppress salt intake. Cell Metabolism *17*, 507-519.
- Sakuraba, J., Shintani, T., Tani, S., and Noda, M. (2013). Substrate specificity of R3 receptor-like protein-tyrosine phosphatase subfamily towards receptor protein-tyrosine kinases. J. Biol. Chem. *288*, 23421-23431.
- Yonehara, K., Farrow, K., Ghanem, A., Hillier, D., Balint, K., Teixeira, M., Jüttner, J., Noda, M., Neve, R.L., Conzelmann, K.-K., and Roska, B. (2013). The first stage of cardinal direction selectivity is localized to the dendrites of retinal ganglion cells. Neuron *79*, 1078-1085.

〔Original paper (E-publication ahead of print)〕

- Unezaki, S., Katano, T., Hiyama, T.Y., Tu, N.H., Yoshii, S., Noda, M., and Ito, S. Involvement of Na_x sodium channel in peripheral nerve regeneration via lactate signaling. Eur. J. Neurosci. 2013 Nov. 29.

〔Review article〕

- Noda, M., and Sakuta, H. (2013). Central regulation of body-fluid homeostasis. Trends Neurosci. *36*, 661-673.