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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

Adenomatous polyposis coli 2 (APC2) 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 growth cone behaviors 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.

Very recently, two siblings with Sotos syndrome-like features caused by homozygous frame shift mutation in the *APC2* gene were found by whole exome sequencing. Sotos syndrome (OMIM #117550) is characterized by varying

degrees of mental retardation and a combination of typical facial features (prominent forehead with a receding hairline, downslanting palpebral fissures, and a pointed chin) and large head circumference its estimated incidence is 1/14,000 live births. Sotos syndrome has been known to be caused by haploinsufficiency in the *NSD1* gene. However, these patients had no mutations in *NSD1* or other known susceptibility genes.

We performed an etiological study on these patients. Cell-based functional assays indicated that the mutant APC2 of these patients was a functionally null protein. *Apc2*-deficient mice exhibited significantly impaired learning and memory abilities, together with an abnormal brain structure and head shape, indicating a high degree of concordance between the phenotypes observed in human patients and knockout mice. We demonstrated for the first time that the expression of *APC2* was under the control of *NSD1*. The impaired migration of cortical neurons was observed when *Nsd1* was knocked down in the embryonic mouse brain, as in the *Apc2*-deficient brain, and this defect was rescued by the forced expression of *Apc2* (Figure 1). Our study explains molecular mechanisms of the intellectual disability associated with Sotos syndrome.

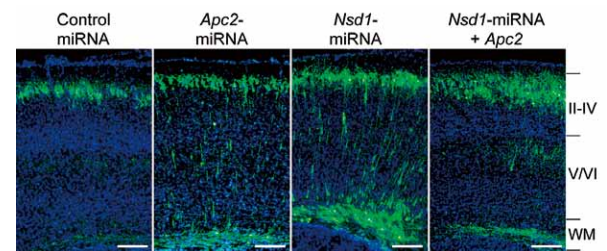


Figure 1. Effects of *Nsd1* knockdown on the migration of cortical neurons during development. The control miRNA, *Apc2*-miRNA, or *Nsd1*-miRNA construct was electroporated into the ventricular region of the forebrain at E15.5. For the rescue experiment, *Nsd1*-miRNA was co-electroporated with an *Apc2*-expressing plasmid. At P7 the location of transfected cells (GFP+) was assessed in coronal sections of the cortex. Nuclei were stained with DAPI. The layers of the cortex are indicated on the right side. WM, white matter. Scale bars, 100 μ m.

II. 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 RPTPs, especially of the R3 and R5 subfamilies.

2-1 R3 RPTP subfamily

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 performed a large scale examination of the enzyme-substrate interaction between the R3 RPTP members and representative RPTKs covering

all RPTK subfamilies. We revealed that multiple RPTKs are recognized as substrates by the R3 RPTPs. Among the enzyme-substrate relationships identified, we examined the interaction between the R3 RPTPs and insulin receptor (IR) in detail.

Co-expression of R3 RPTPs with the IR in HEK293T cells suppressed the insulin-induced tyrosine phosphorylation of the IR. *In vitro* assays using synthetic phosphopeptides revealed that R3 RPTPs preferentially dephosphorylates particular phosphorylation sites of the IR; Y960 in the juxtamembrane region and Y1146 in the activation loop. It has been revealed that phosphorylation of Y1146 and Y960 are required for the full activation and the signal transduction of the IR, respectively. Therefore, R3 RPTPs are thought to effectively dephosphorylate these sites of IR, and thereby suppress insulin signaling.

RT-PCR and *in situ* hybridization showed that only PTPRJ was expressed together with IR in major insulin target tissues, such as skeletal muscle, liver, and adipose tissue. Expectedly, *Ptprj*-deficient mice exhibited enhanced activation of IR and Akt by insulin, and improved glucose and insulin tolerance (Figure 2). These results indicate that PTPRJ is a physiological enzyme attenuating insulin signaling *in vivo*, and selective inhibitors of PTPRJ could be insulin-sensitizing agents.

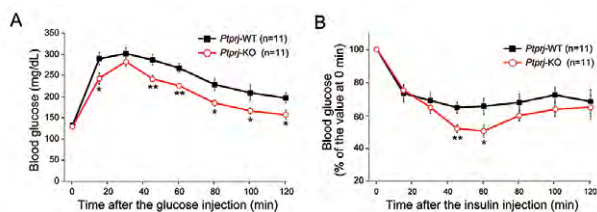


Figure 2. Improved glucose and insulin tolerance in *Ptprj*-KO mice. In glucose (A) and insulin (B) tolerance tests, glucose (2 g/kg body weight) and insulin (1 unit/kg body weight) were administered by intraperitoneal injection to fasted mice, respectively, and blood glucose levels were assayed. Values are shown as the mean \pm SEM. The asterisks indicate significant differences from WT mice by Student's *t*-test (* p < 0.05, ** p < 0.01).

2-2 R5 RPTP subfamily

Protein-tyrosine phosphatase receptor type Z (PTPRZ) is predominantly expressed in glial and neuronal cells during development in the central nervous system (CNS). Oligodendrocyte precursor cells (OPCs) are the principal source of myelinating oligodendrocytes. Deficiencies in myelination in diseases such as multiple sclerosis (MS) lead to serious neurological disorders. Two animal disease models have been widely accepted for studying the clinical and pathological features of MS lesions. Experimental autoimmune encephalomyelitis (EAE) is a T-cell-mediated inflammatory CNS demyelination model, and the cuprizone model of demyelination is induced by a T-cell-independent mechanism through feeding of the copper chelator cuprizone. We previously showed that adult *Ptprz*-deficient mice were less susceptible to the induction of EAE than wild-type controls.

This year, we revealed that cuprizone-fed *Ptprz*-deficient mice exhibited severe demyelination and axonal damage

in the corpus callosum, similar to wild-type mice, whereas remyelination in *Ptprz*-deficient mice after cuprizone-induced demyelination was significantly accelerated. Accelerated remyelination was attributed to the higher differentiation potential of OPCs, because the number of oligodendrocyte-lineage cells recruited to the demyelinated area was not altered. Importantly, pleiotrophin, one of the inhibitory ligands for PTPRZ, was transiently expressed in the brain upon demyelination, and gradually disappeared with remyelination. Pleiotrophin was detected in affected cortex neurons and their axon fibers in the cuprizone model. Since pleiotrophin colocalized with a synaptic vesicle marker, the relevant neurons are presumed to release pleiotrophin from their demyelinated axons (Figure 3). The treatment of a primary culture of wild-type mouse brain cells with pleiotrophin itself did not induce oligodendrocyte maturation, but enhanced thyroid hormone-induced oligodendrocyte differentiation. Of note, the differentiation of *Ptprz*-deficient cells was not further potentiated by pleiotrophin. Pleiotrophin released from demyelinated fibers may stimulate the differentiation of OPCs recruited in the demyelinated area *in vivo*.

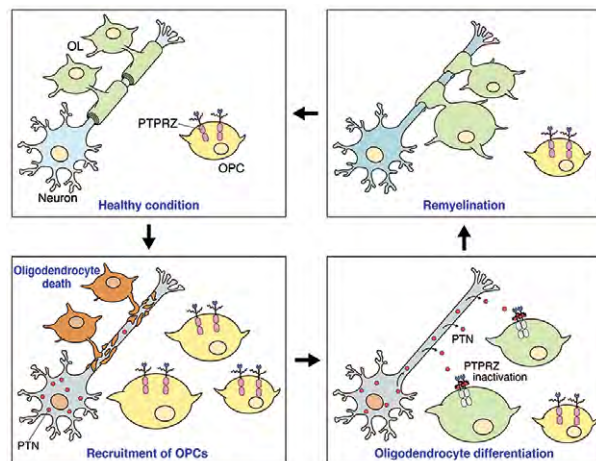


Figure 3. Proposed mechanism for remyelination after demyelinating lesions. OPCs, but not mature oligodendrocytes (OLs), abundantly express PTPRZ-A/B receptor proteins as chondroitin sulfate (CS) proteoglycans. The CS moiety of PTPRZ is important for achieving the high-affinity binding of PTN to the core protein. PTPRZ activity is requisite to maintaining the immature state of OPCs. After cuprizone-induced oligodendrocyte death and demyelination, the expression of PTN is transiently upregulated in damaged neurons. PTN may be released from demyelinated axons and bind to PTPRZ at the cell surface of OPCs that are recruited to lesions, probably through PTPRZ-independent mechanisms. The binding of PTN results in receptor dimerization or oligomerization, thereby inhibiting its catalytic activity. PTPRZ inactivation releases the block of differentiation in OPCs, so the remyelination of neighboring axons is initiated.

We established immature oligodendrocytes (OL1 cells) from *p53*-deficient mice. They were strongly positive for the two receptor isoforms of PTPRZ-A and PTPRZ-B with chondroitin sulfate chains; however, their expression gradually decreased with differentiation, with only PTPRZ-B being weakly detectable in mature oligodendrocytes. The treatment of immature OL1 cells with pleiotrophin enhanced the phosphorylation of p190 RhoGAP, which is a substrate molecule of PTPRZ. We found that pleiotrophin reduced the expression of NG2 proteins in OL1 cells. Therefore, it is

conceivable that the catalytic activity of PTPRZ functions to maintain OPCs in an undifferentiated state, and the pleiotrophin-induced inactivation of PTPRZ releases this blockage (Figure 3).

III. 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 ($\text{Na}_v 1.1-1.9$), is a Na^+ -concentration ($[\text{Na}^+]$)-sensitive Na channel with a gating threshold of ~ 150 mM for extracellular $[\text{Na}^+]$ ($[\text{Na}^+]_o$) *in vitro*. Na_x is preferentially expressed in glial cells of sensory circumventricular organs including the subformal organ, and is involved in $[\text{Na}^+]$ sensing for the control of salt-intake behavior.

Although Na_x is also expressed in neurons of some brain regions including the amygdala and cerebral cortex, the channel properties of Na_x in neurons have not yet been adequately characterized. To investigate the properties of Na_x expressed in neurons, we established an inducible cell line of Na_x using the mouse neuroblastoma cell line, Neuro-2a, which is endogenously devoid of the expression of Na_x (N2a-Mf1 cell). Functional analyses of this cell line revealed that the $[\text{Na}^+]$ -sensitivity of Na_x in neuronal cells was similar to that expressed in glial cells.

Furthermore, we found that Na_x bound to postsynaptic density protein 95 (PSD95) through its PSD95/Disc-large/ZO-1 (PDZ)-binding motif at the C-terminus. Na_x co-localized with PSD95 clusters along dendrites of lateral amygdala

neurons. PSD95 is known as an anchoring and scaffolding protein for receptors and other postsynaptic proteins. The interaction between Na_x and PSD95 may be involved in promoting the surface expression of Na_x channels by suppressing their endocytosis, because the depletion of endogenous PSD95 resulted in a decrease in Na_x at the plasma membrane (Figure 4). These results show for the first time that Na_x functions as a $[\text{Na}^+]$ -sensitive Na channel in neurons as well as in glial cells.

Publication List:

[Original papers]

- Almuriekh, M.*, Shintani, T.*, Fahiminiya, S.*, Fujikawa, A., Kuboyama, K., Takeuchi, Y., Nawaz, Z., Nadaf, J., Kamel, H., Kitam, A.K., Samiha, Z., Mahmoud, L., Ben-Omran, T., Majewski, J., and Noda, M. (2015). Loss-of-function mutation in *APC2* causes Sotos Syndrome features. *Cell Reports* 10, 1585-1598. *Co-first authors.
- Fujikawa, A., Matsumoto, M., Kuboyama, K., Suzuki, R., and Noda, M. (2015). Specific dephosphorylation at Tyr-554 of *Git1* by *Ptpz* promotes its association with *Paxillin* and *Hic-5*. *PLoS One* 10, e0119361.
- Kuboyama, K., Fujikawa, A., Suzuki, R., and Noda, M. (2015). Inactivation of protein tyrosine phosphatase receptor type Z by pleiotrophin promotes remyelination through activation of differentiation of oligodendrocyte precursor cells. *J. Neurosci.* 35, 12162-12171.
- Matsumoto, M., Hiyama, T.Y., Kuboyama, K., Suzuki, R., Fujikawa, A., and Noda, M. (2015). Channel properties of Na_x expressed in neurons. *PLoS One* 10, e0126109.
- Shintani, T., Higashi, S., Takeuchi, Y., Gaudio, E., Trapasso, F., Fusco, A., and Noda, M. (2015). The R3 receptor-like protein-tyrosine phosphatase subfamily inhibits insulin signaling by dephosphorylating the insulin receptor at specific sites. *J. Biochem.* 158, 235-243.
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[Review articles]

- Noda, M., and Hiyama, T.Y. (2015). The Na_x channel: What it is and what it does. *Neuroscientist* 21, 399-412.
- Noda, M., and Hiyama, T.Y. (2015). Sodium sensing in the brain. *Pflügers Arch. – Eur. J. Physiol.* 467, 465-474.

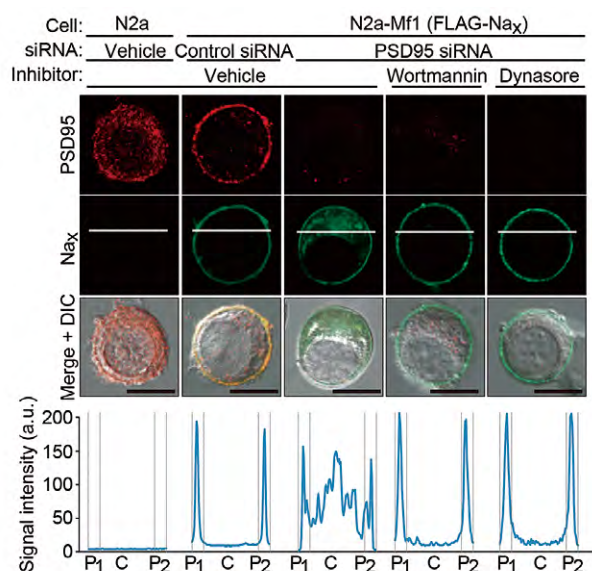


Figure 4. Subcellular distribution of Na_x in non-treated Neuro-2a (N2a) cells, or N2a-Mf1 cells transfected with control or *PSD95* siRNA. Upper panels: Immunostaining with anti-PSD95 and anti- Na_x antibodies. In order to inhibit endocytosis, cells expressing Na_x were treated with 100 nM wortmannin or 200 μM dynasore for 6 h. Scale bars, 10 μm . Lower graphs: Fluorescence intensity profiles along the white lines in the upper panels. The profile was divided into three parts (P1 : C : P2 = 15 : 70 : 15 in length). a.u., arbitrary unit.