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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 the visual systems of chicks and 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 a fundamental feature 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 axon 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. APC2 is distributed along microtubules in growth cones as well as axon shafts of retinal axons. 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.

Recently, we generated *Apc2*-deficient mice by a gene-targeting technique. In the homozygous mutant mice, robust defects in neuronal lamination were observed in the cortex (Figure 1A), hippocampus, cerebellum (Figure 1B), and olfactory bulb. *In vivo* BrdU labeling and immunohistochemical analyses with specific markers revealed that the laminary abnormalities are a result of dysregulated neuronal migration by a cell-autonomous mechanism. Dissociated *Apc2*-deficient cerebellar granule cells showed no obvious alterations in migration under non-stimulated conditions, however, Brain-derived neurotrophic factor (BDNF)-stimulated directional migration was perturbed.

Total internal reflection fluorescence (TIRF) microscopy revealed that APC2 is distributed along actin fibers as well as microtubules. BDNF-stimulated F-actin formation at the leading edge was impaired in migrating *Apc2*-deficient neurons, along with dysregulation of Rho GTPase activity. Thus APC2 is an essential mediator of the cytoskeletal regulation at leading edges in response to extracellular signals. The phenotypes observed in *Apc2*-deficient mice suggest that mutations in *APC2* in humans may cause a neurodevelopmental disorder.

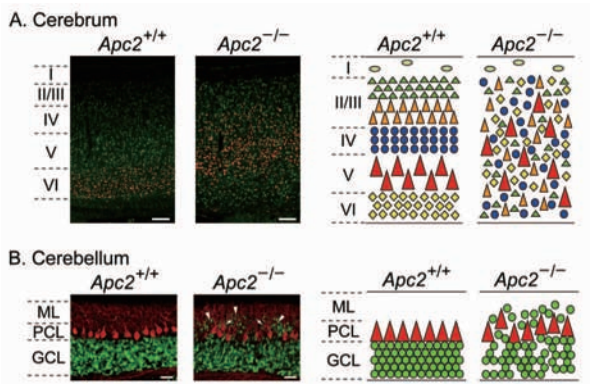

Figure 1. Laminary defects in the brain of *Apc2*-deficient mice. **A**, Sagital sections of P30 cerebral cortex stained with anti-NeuN (green, a neuron-specific marker) and anti-FoxP2 (red, a polymorphous cell-specific marker). The cerebral cortex of the wild-type (*Apc2*$^{+/+}$) mouse is organized into six layers and FoxP2-positive cells are mainly distributed in layer VI. In the *Apc2*-deficient mouse (*Apc2*$^{-/-}$), however, they are observed broadly in layers II to V. Right panels are schematic drawings of distribution patterns of neuronal subtypes in the wild-type and *Apc2*-deficient mouse. In the *Apc2*-deficient mouse, cortical layers were poorly organized and layer boundaries are blurred and indistinct. Scale bars, 50 μm. **B**, Sagital sections of P20 cerebellum stained with anti-NeuN (green, a granule cell-specific marker) and anti-calbindin D-28K (red, a Purkinje cell-specific marker). Arrows and arrowheads indicate ectopically distributed Purkinje and granule cells, respectively. Right panels are schematic drawings of distribution patterns of neuronal subtypes in the wild-type and *Apc2*-deficient mouse. ML, molecular layer; PCL, Purkinje cell layer; GCL, granule cell layer. Scale bars, 50 μm.

II. Regeneration of retinal axons

CNS neurons in fish can regrow their axons after nerve transection, while CNS neurons in mammals lose their capacity to regenerate. Thus, we have been investigating genes involved in optic nerve regeneration using fish visual systems. We found that expression of coagulation factor XIII

A subunit (FXIII-A), a protein cross-linking enzyme, is upregulated in the goldfish optic nerve and retina during regeneration. The cells producing FXIII-A were astrocytes/microglial cells and retinal ganglion cells (RGCs) in the optic nerve and retina, respectively. Overexpression of FXIII-A in RGCs and addition of extracts of optic nerves with injury to retinal explants induced significant neurite outgrowth from the retina. From these observations, we concluded that the increase of FXIII in RGCs promotes neurite sprouting from injured RGCs, whereas the increase of FXIII in optic nerves facilitates elongation of regrowing axons.

III.Physiological roles of protein tyrosine phosphatase receptor type Z

Protein-tyrosine phosphatase receptor type Z (Ptprz, also known as PTPζ/RPTPβ) is a member of the R5 receptor-like protein tyrosine phosphatase (RPTP) subfamily. Ptprz is predominantly expressed in glial and neuronal cells in the central nervous system (CNS) and its physiological importance has been demonstrated through studies with *Ptprz*-deficient mice. Ptprz modulates hippocampal synaptic plasticity: adult *Ptprz*-deficient mice display impairments in spatial and contextual learning. Ptprz is expressed also in the stomach, where it is used as a receptor for VacA, a cytotoxin secreted by *Helicobacter pylori*: *Ptprz*-deficient mice are resistant to gastric ulcer induction by VacA.

This year, we revealed that Ptprz plays a negative role in oligodendrocyte differentiation in early CNS development and remyelination in demyelinating CNS diseases, through the dephosphorylation of its substrates such as p190RhoGAP. We first found an early onset of the expression of myelin basic protein (MBP), a major protein of the myelin sheath, and early initiation of myelination *in vivo* during development of the *Ptprz*-deficient mouse, as compared with the wild-type (Figure 2A). In addition, oligodendrocytes appeared earlier in primary cultures from *Ptprz*-deficient mice than wild-type mice.

We subsequently found that adult *Ptprz*-deficient mice are less susceptible to experimental autoimmune encephalomyelitis (EAE) induced by active immunization with myelin/oligodendrocyte glycoprotein (MOG) peptide than were wild-type mice. However, the number of T-cells and macrophages/microglia infiltrating into the spinal cord were not decreased in *Ptprz*-deficient mice after MOG immunization, suggesting that the reduced tissue damage is not attributable to an inhibition of infiltration by inflammatory cells.

It is known that Fyn tyrosine kinase-mediated down-regulation of Rho activity through activation of p190RhoGAP is crucial for oligodendrocyte differentiation and myelination. Here, p190RhoGAP is one of the physiological Ptprz substrates. After EAE induction, the tyrosine phosphorylation of p190RhoGAP increased significantly, and the EAE-induced loss of MBP was markedly suppressed in the white matter of the spinal cord in *Ptprz*-deficient mice (Figure 2B).

Thus, selective inhibition of Ptprz signaling could be an effective and plausible therapeutic strategy for treating demyelinating diseases.

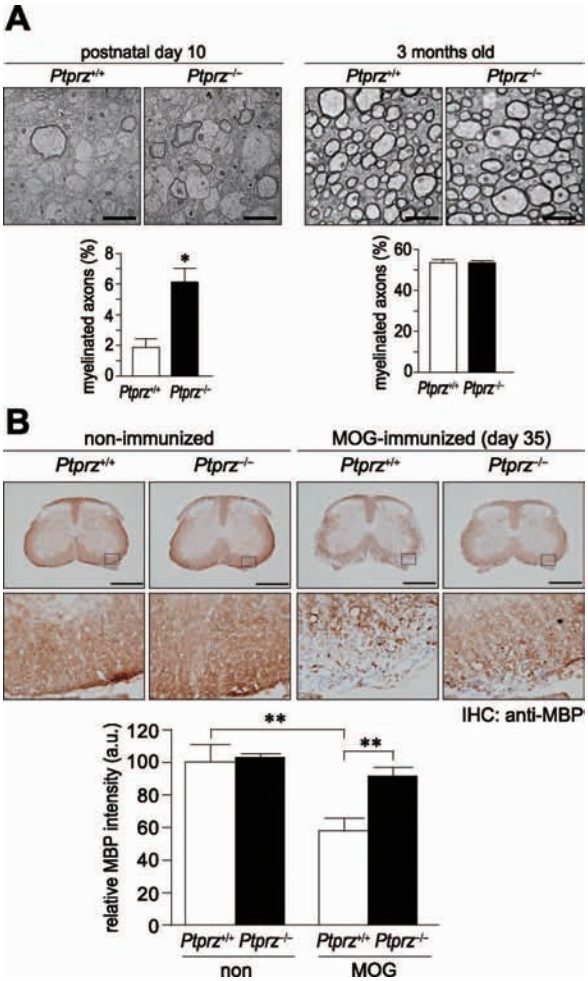

Figure 2. Ptprz plays a negative role in oligodendrocyte differentiation in early CNS development and remyelination in demyelinating CNS diseases. **A**, Early myelination in *Ptprz*-deficient mice. Electron micrographs of transverse sections at the corpus callosum from mice at postnatal day 10 (left), and 3 months old (right). Scale bars, 2 μm. Percentages of myelinated axons in total axons are shown at the lower position of each panel. Data are the mean ± SEM $*p < 0.05$. **B**, Reduced MBP loss in *Ptprz*-deficient mice after EAE induction. Anti-MBP staining of the spinal cord sections from wild-type and *Ptprz*-deficient mice 35 days after MOG immunization, or non-immunized control mice. The lower images are enlargements of the areas enclosed by squares in the upper images. Scale bars, 500 μm. MBP signals are expressed as the relative change compared with the non-immunized wild-type mice, and shown at the bottom. Data are the mean ± SEM. $**p < 0.01$.

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 with a threshold of ~150 mM for extracellular Na^+ concentration $[Na^+]_o$ *in vitro*.

In the brain, Na_x channels are specifically 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.

Na_x has a putative PSD-95/Disc-large/ZO-1 (PDZ)-binding motif at the carboxyl-terminus. We thus hypothesized that the Na_x channel may be regulated by PDZ-scaffold proteins. Very recently, we found that several PDZ proteins bind to Na_x by PDZ-array overlay assay. Among them, synapse-associated protein 97 (SAP97/DLG1) was coexpressed with Na_x in the SFO. In C6 glioblastoma cells, destruction of the PDZ-binding motif of Na_x, Na_x(T1679A), resulted in a decrease in cell-surface Na_x, which was attenuated with inhibitors of endocytosis (Figure 3A). Depletion of SAP97 also led to the reduction in the surface expression of wild-type Na_x (Figure 3B). Next, functional relevance of the binding of Na_x with SAP97 was confirmed by Na^+-imaging studies (Figure 3C). When the extracellular Na^+ concentration, $[Na^+]_o$ was increased from 145 mM to 170 mM, both C6M16 cells expressing wild-type Na_x and C6M(TA)8 cells expressing the Na_x(T1679A) mutant showed increases in the intracellular Na^+ concentration, $[Na^+]_i$, and the level eventually reached the same equilibrium point between Na^+ influx by Na_x and Na^+ export by Na^+/K^+-ATPase. However, importantly, C6M(TA)8 cells took longer to reach this equilibrium level than control C6M16 cells. This is probably because the reduction in the number of surface Na_x channels diminished the Na^+ influx. Thus, SAP97 appears to play an important role in sensing body-fluid $[Na^+]$ in the SFO through regulation of the surface expression of the sensor channel.

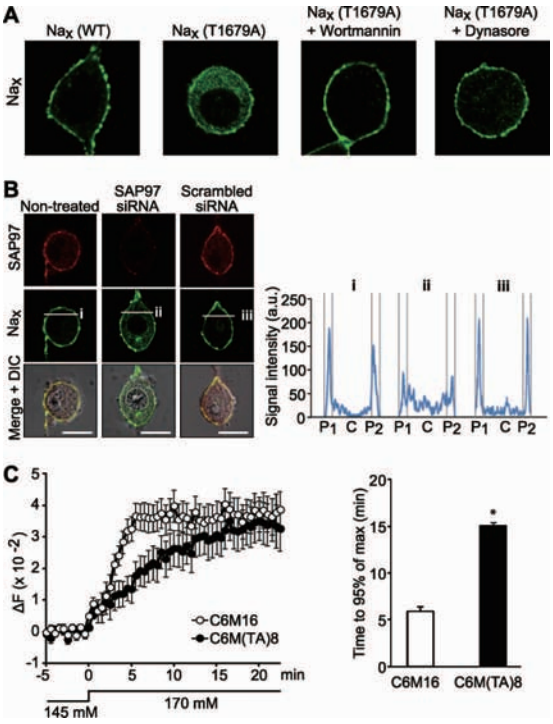

Figure 3. SAP97 promotes the stability of Na_x channels at the plasma membrane. **A**, Decrease in cell-surface expression of the Na_x mutant with Thr-1679 changed to Ala (mutation in the PDZ-binding motif) in C6 cells and its improvement by treatment with endocytosis inhibitors, wortmannin and dynasore. After the induction of the expression of Na_x(T1679A) channels, cells were treated with 100 nM wortmannin or 200 μM dynasore for 6 h. Then the cells were fixed, permeabilized, and stained with anti-Na_x. **B**, Reduction in the cell-surface expression of Na_x with depletion of SAP97. Non-treated C6M16 cells, or C6M16 cells transfected with siRNA for SAP97 or scrambled siRNA were immunostained with anti-SAP97 and anti-Na_x. The fluorescence intensity profiles along the white lines are shown in the right panel. Scale bars, 10 μm. a.u., arbitrary unit. **C**, Reduced sodium influx in C6 cells in the absence of any association between Na_x and SAP97. Left: Na^+ imaging of C6M16 (wild-type Na_x) and C6M(TA)8 (T1679A mutant Na_x) cells upon elevation of the extracellular Na^+ concentration from 145 mM to 170 mM. The coordinate gives the fluorescence ratio (ΔF340/380 nm) in Na^+ imaging with sodium-binding benzofuran isophthalate acetoxymethyl ester (SBFI/AM), representing the intracellular Na^+ concentration. The physiological 145 mM Na^+ solution was changed to a 170 mM solution at 0 min. Right: Summary of the time to reach 95% of the maximum fluorescence ratio. Data represent the mean ± SE (n = 40 for each); $*P < 0.01$, two-tailed *t* test.

Publication List

〔Original papers〕

- Kuboyama, K., Fujikawa, A., Masumura, M., Suzuki, R., Matsumoto, M., and Noda, M. (2012). Protein tyrosine phosphatase receptor type Z negatively regulates oligodendrocyte differentiation and myelination. PLoS ONE *7*, e48797.
- Matsumoto, M., Fujikawa, A., Suzuki, R., Shimizu, H., Kuboyama, K., Hiyama, T.Y., Hall, R.A., and Noda, M. (2012). SAP97 promotes the stability of Na_x channels at the plasma membrane. FEBS Lett. *586*, 3805-3812.
- Shintani, T., Takeuchi, Y., Fujikawa, A., and Noda, M. (2012). Directional neuronal migration is impaired in mice lacking adenomatous polyposis coli 2. J. Neurosci. *32*, 6468-6484.
- Sugitani, K., Ogai, K., Hitomi, K., Nakamura-Yonehara, K., Shintani, T., Noda, M., Koriyama, Y., Tanii, H., Matsukawa, T., and Kato, S. (2012). A distinct effect of transient and sustained upregulation of cellular factor XIII in the goldfish retina and optic nerve on optic nerve regeneration. Neurochem. Int. *61*, 423-432.