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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. We are now investigating a mutation in human *APC2* gene identified in patients with intellectual disabilities.

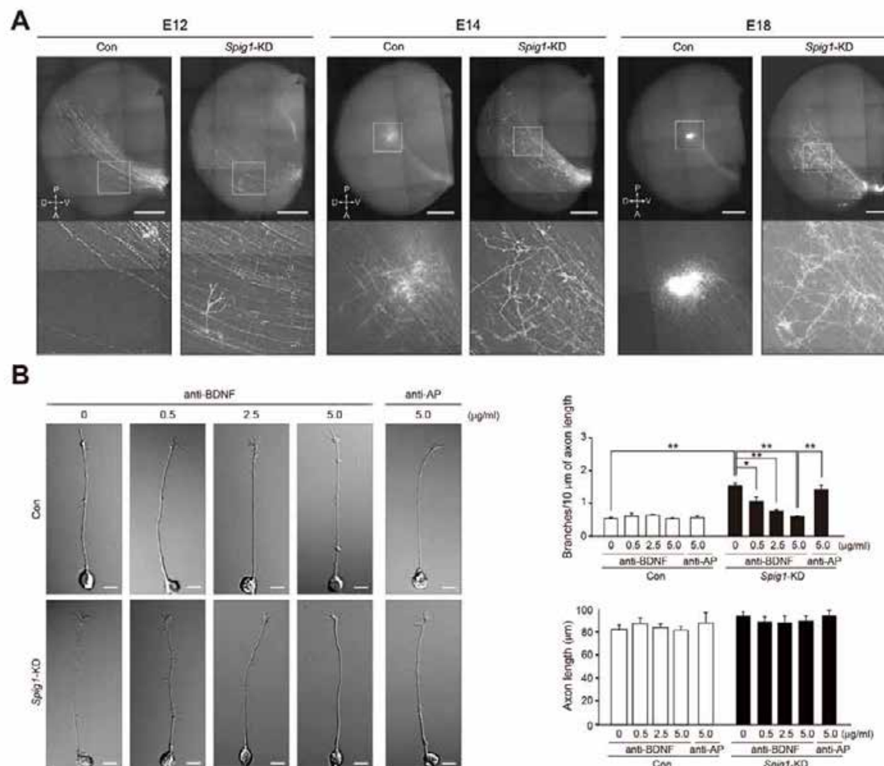

Figure 1. Effect of *Spig1*-knockdown (KD) in the developing chick retina. **A**, Projection pattern of dorsal RGC axons by DiI labeling in control (Con) and *Spig1*-KD embryos. Higher magnification of the boxed area is shown in the lower part of each panel. A, anterior; P, posterior; D, dorsal; V, ventral. Scale bars, 1 mm. **B**, Effect of a neutralizing antibody for BDNF on the elongation and branching of RGC axons. After the electroporation of *Spig1*-shRNA or control retroviral construct at HH stage 9-10, retinal cells were dissociated from the dorsal one-third of the retina at E8. The branch number and axon length of RGC axons were quantified. Data are shown as the mean ± SE of four independent experiments. *$p < 0.05$, **$p < 0.001$ (ANOVA with Scheffé's *post hoc* tests). Scale bars, 10 μm.

Note: Those members appearing in the above list twice under different titles are members whose title changed during 2014. The former title is indicated by an asterisk (*).

Another molecule is SPARC-related protein containing immunoglobulin domains 1 (SPIG1, also known as Follistatin-like protein 4), which is a secretory protein expressed in a dorsal-specific manner in the developing chick retina. The knockdown of *Spig1* in the retinal ganglion cells (RGCs) of developing chick embryos induced robust ectopic branching of dorsal RGC axons and failed to form a tight terminal zone at the proper position on the tectum (Figure 1A). The knockdown of *Spig1* in RGCs also led to enhanced axon branching *in vitro*. However, this was canceled by the addition of a neutralizing antibody against brain-derived neurotrophic factor (BDNF) to the culture medium (Figure 1B). SPIG1 and BDNF were colocalized in vesicle-like structures in cells. SPIG1 bound with the proform of BDNF (proBDNF) but very weakly with mature BDNF *in vitro*. The expression and secretion of mature BDNF were significantly decreased when SPIG1 was exogenously expressed with BDNF in HEK293T or PC12 cells. The amount of mature BDNF proteins as well as the tyrosine phosphorylation level of the BDNF receptor, tropomyosin-related kinase B (TrkB), in the hippocampus were significantly higher in *Spig1*-knockout mice than in wild-type mice (Figures 2A and B). Furthermore, the spine density of CA1 pyramidal neurons was consistently increased (Figure 2C). Together, these results suggest that SPIG1 negatively regulated BDNF maturation by binding to proBDNF, thereby suppressing axonal branching and spine formation.

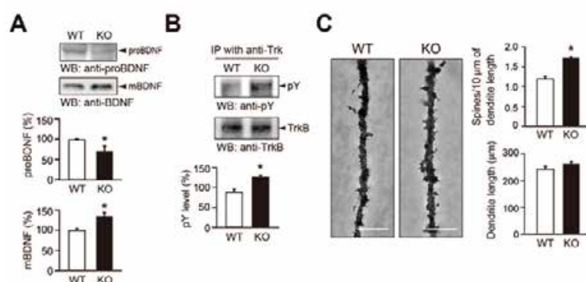

Figure 2. Functional interactions between SPIG1 and BDNF in hippocampal cells. **A**, Comparison of *BDNF* expression in the hippocampus of wild type (WT) and *Spig1*-knockout (KO) mice at postnatal day 14 (P14). The amounts of BDNF are presented by densitometric units normalized to the value for the WT. Data are shown as the mean ± SE (n = 14 for each). *p < 0.05 (Student's t test). **B**, Increased tyrosine phosphorylation of TrkB in the hippocampus of *Spig1*-KO mice. The tyrosine phosphorylation of TrkB proteins immunoprecipitated with an anti-Trk antibody from the hippocampus at P14 was analyzed using 4G10 and anti-TrkB. Densitometric data are presented as a percentage of the WT control (bottom). Data are mean ± SE (WT, n = 6; *Spig1*-KO, n = 8). *p < 0.05 (Student's t test). **C**, Golgi staining of the hippocampal tissues from WT and *Spig1*-KO mice at P10. We analyzed the dendritic segments of CA1 pyramidal neurons. Spine density and dendritic length are shown as the mean ± SE (n = 6 for each). *p < 0.01 (Student's t test). Scale bars, 10 ㎜.

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 the R3 and R5 subfamilies of RPTPs.

2-1 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 performed a large scale examination of the enzyme-substrate interaction between the R3 RPTP members and representative RPTKs covering RPTK subfamilies. We revealed that multiple RPTKs are recognized as substrates by the R3 RPTPs. We also demonstrated that the R3 members showed differences in substrate specificity toward individual RPTKs. On the basis of the enzyme-substrate relationships identified, we are now investigating the physiological roles of the R3 RPTP subfamily by using their knockout mice.

2-2 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.

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 (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. The threshold value of Na_x for $[Na^+]_o$ was ~150 mM *in vitro*. In the SFO, however, endothelin-3 (ET-3) shifts the $[Na^+]_o$

dependency of Na_x activation to the lower-concentration side in a dose-dependent manner. This shift enables Na_x to gate even when $[Na^+]_o$ is in the physiological range (135–145 mM). Na_x thus functions as the brain's Na^+-level sensor for the homeostatic control of $[Na^+]$ in body fluids.

We can summarize the cellular mechanisms for $[Na^+]$-sensing and $[Na^+]$-dependent regulation of neural activities in the SFO as presented in Figure 3. The sensory CVOs, including the SFO, are characterized by the extensive networks of fenestrated capillaries which allow ingredients of plasma to release to the intercellular space. Their ventricular side is partitioned by an ependymal cell layer facing the third ventricle. Na_x channels populate perineural processes of astrocytes and ependymal cells in the SFO. Even under hydrated (normal) conditions, ET-3 level expressed in the SFO can modulate the $[Na^+]_o$ dependency of Na_x and make Na_x sensitive to an increase in $[Na^+]_o$ in the physiological range. When animals are dehydrated, $[Na^+]$ in plasma and CSF significantly increases above the usual level. Under such conditions, the $[Na^+]_o$ exceeds the threshold of Na_x, Na_x channels open, and the $[Na^+]_i$ in these Na_x-bearing cells is increased. This leads to activation of Na^+/K^+-ATPase in these cells. Activated Na^+/K^+-ATPase consumes ATP higher than the usual level to pump out Na^+. To fuel Na^+/K^+-ATPase with ATP, the glial cells enhance glucose uptake to stimulate anaerobic glycolysis. Lactate, the end product of the anaerobic glycolysis, is released from the glial cells and supplied to neurons, including GABAergic neurons, through the processes enveloping them. Lactate stimulates the activity of the GABAergic neurons through production of ATP, which presumably leads to the regulation of hypothetic neurons involved in the control of salt-intake behavior. In dehydrated Na_x-deficient mice, the $[Na^+]$-dependent stimulation of glycolysis is impaired and the activity of the GABAergic neurons is not promoted.

Na_x is also expressed in non-myelinating Schwann cells of the adult peripheral nervous system, but the pathophysiological role remained unclear. Recently, we found that functional recovery of the hind paw responses from the sciatic nerve transection was delayed in Na_x-deficient mice. Our studies revealed that Na_x is 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.

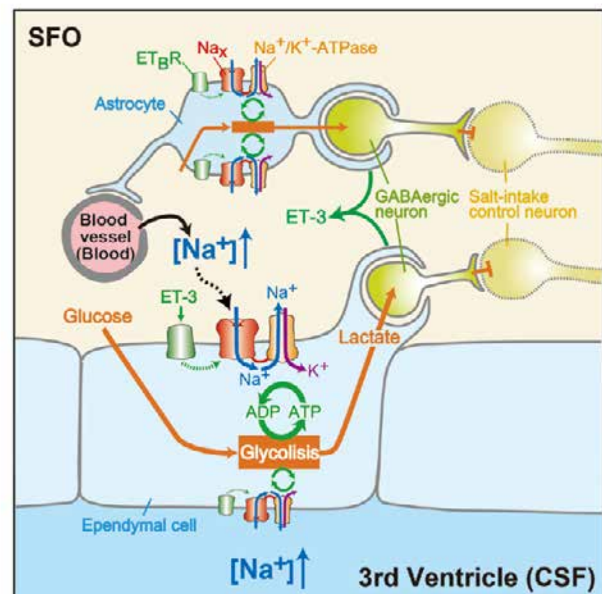

Figure 3. Overview of the $[Na^+]$-sensing mechanism and Na_x-dependent regulation of neuronal activity in the SFO.

Publication List

〔Original papers〕

- Suzuki, R., Matsumoto, M., Fujikawa, A., Kato, A., Kuboyama, K., Shintani, T., Sakuta, H., and Noda, M. (2014). SPIG1 negatively regulates BDNF maturation. J. Neurosci. *34*, 3443-3453.
- Unezaki, S., Katano, T., Hiyama, T.Y., Tu, N.H., Yoshii, S., Noda, M., and Ito, S. (2014). Involvement of Na_x sodium channel in peripheral nerve regeneration via lactate signaling. Eur. J. Neurosci. *39*, 720-729.

〔Review article〕

- Noda, M., and Hiyama, T.Y. (2014). The Na_x channel: What it is and what it does. The Neuroscientist DOI: 10.1177/1073858414541009.