Division of Molecular Neurobiology (Prof. M. Noda)

1. Research activity in the past 10 years

(Numbers in parentheses represent original articles and those underlined represent reviews or books in the List of Publication)

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, terminal differentiation of neurons, navigation, branching and targeting of axons, and formation, refinement and plasticity of synapses. 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.

Our main research themes are: (A) Molecular mechanisms for retinal patterning and topographic retinotectal projection, (B) Functional roles of receptor-type protein tyrosine phosphatases (RPTPs) mainly in the brain, and (C) Mechanisms for body-fluid homeostasis in the mammalian brain. Achievements of respective research groups during the last ten years are summarized below.

A. Molecular mechanisms for the specificity in neural connections

(1) Mechanisms for the regional specification in the developing retina

Retinal patterning which determines region-specific features of retinal ganglion cells (RGCs) is the basis for the topographic retinotectal projection. To understand the molecular mechanisms underlying the retinal patterning during development, an overall view of asymmetrically expressed molecules in the developing retina is needed. We performed a large-scale screening of the E8 chick retina using the restriction landmark cDNA scanning (RLCS) technique (13), in addition to a cDNA subtractive hybridization technique (Nature *382*, 632-635, 1996). We identified 33 asymmetrically distributed molecules along the nasotemporal (N-T) axis and 20 along the dorsoventral (D-V) axis in the embryonic retina: Some showed a double-gradient expression pattern.

In the past ten years, we have been devoting our efforts to the characterization of the individual functional roles of these molecules, and to the determination of the cascades controlling their expression. To this end, we developed and took advantage of a retrovirus vector-mediated gene transfer into the chick optic vesicle by *in ovo* electropolation (4, 5). We can now present gene cascades of the topographic molecules for retinal patterning and retinotectal projection (**Figure 1**; modified from ref. 25; see also 6).

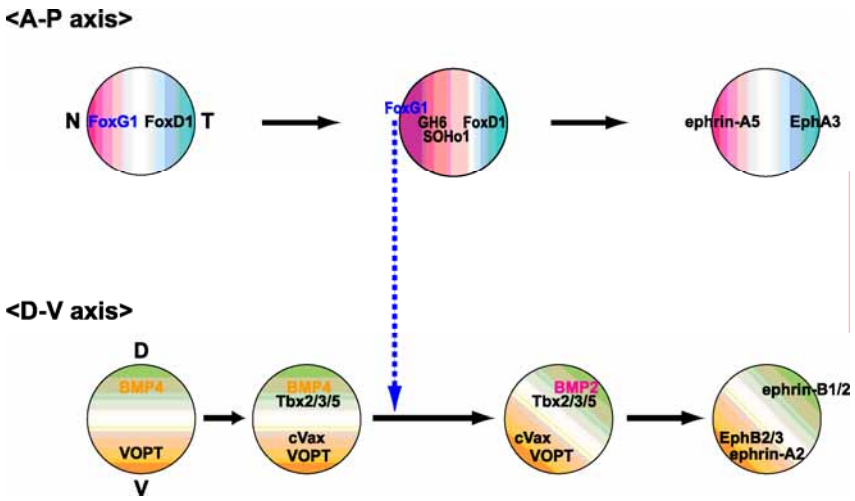

Figure 1. Developmental expression of topographic molecules in the chick retina. *FoxG1* and *FoxD1* are expressed in a counter-gradient manner in the optic vesicle. After the optic cup forms, the expression domains of *FoxG1* and *FoxD1* are separated by reducing their positive regions to a narrow region of the nasal or temporal retina, respectively. Subsequently, *GH6* and *SOHo1* are expressed specifically in the nasal retina from HH stage 12-14. The expression domains of *GH6* and *SOHo1* fill the gap between the expression areas of *FoxG1* and *FoxD1*. *EphA3* and *ephrin-A5* are homogeneously expressed in the retina at early developmental stages and become restricted to the temporal and nasal retina, respectively, under the control of these transcription factors at later stages. The counteraction between *BMP4* and *Ventroptin* (VOPT) governs the regional specification in the retina along the D-V axis early in development (HH stage 11 to E5). At around E5, *BMP4* expression in the dorsal retina rapidly begins to disappear, and *Ventroptin* comes to be expressed in an oblique-gradient manner from E6 onward (in a V/N-high gradient). Concomitantly, *BMP2* begins to be expressed in a pattern complementary to that of *Ventroptin* (in a D/T-high gradient). The inhibitory action of FoxG1 on BMP signaling is attributable to the change in the expression of *Ventroptin* and *BMP2* to the oblique-gradient pattern. The counteraction between *Ventroptin* and *BMP2* determines the expression of the formerly dorsoventrally-asymmetric molecules and *ephrin-A2* in an oblique-gradient fashion, and resultantly governs the retinal patterning and retinotectal projection along both the A-P and D-V axes. *Ephrin-A2* begins to be expressed much later than *ephrin-A5* from E6 in an oblique-gradient manner from the beginning under the control of the BMP2 signal. Development proceeds from left to right.

Along the A-P axis, fibroblast growth factor (Fgf) and Wnt first play pivotal roles in inducing the expression of two winged-helix transcription factors *FoxG1* (CBF-1) and *FoxD1* (CBF-2) in the

nasal (N) and temporal (T) regions in the optic vesicle, respectively (34). The region-specific expression of *FoxG1* and *FoxD1* begins at an early stage (peaking at E3) and determines the regional specificity in the retina (10, 34; see also, Nature *382*, 632-635, 1996; Dev. Growth. Diff. *41*, 575-587, 1999). Two homeobox transcription factors, *SOHo1* and *GH6*, are consequently expressed specifically in the nasal region. Afterwards, *ephrin-A5* and *ephrin-A2* begin to show nasal-high expression and *EphA3* temporal-high expression in the retina. Misexpression of *FoxG1* in the temporal retina represses the expression of *EphA3* and *FoxD1*, and induces that of *SOHo1*, *GH6*, *ephrin-A5,* and *ephrin-A2* (10). *SOHo1* and *GH6* repress the expression of *FoxD1* and *EphA3* (34). In this process, intriguingly, *FoxG1* controls *ephrin-A5* via a DNA binding-dependent mechanism (as a transcriptional repressor), and *ephrin-A2* via a DNA binding-independent mechanism (as an inhibitor of BMP2 signaling) (10, 25, 34) (see below). In contrast, FoxD1 acts as a transcriptional repressor in controlling its downstream targets (34).

Along the D-V axis, counteraction between BMP4 and Ventroptin first governs the regional specification in the retina (Science *293*, 767-778, 2001): At the early stages of development from E2 to E5, dorsally-expressed *BMP4* determines the regional specificity of the dorsal retina and ventrally-expressed *Ventroptin* counteracts the activity of BMP4 (10). Transcription factors *Tbx2/3/5* in the dorsal retina and *cVax* in the ventral retina begin to be expressed from E3 under the control of the BMP4 signal (25). At approximately E5, *BMP4* expression in the dorsal retina rapidly disappears (10). Concomitantly, *Ventroptin* comes to be expressed in an oblique-gradient fashion (V/N-high pattern from E6 onward). Then, instead of *BMP4*, *BMP2* begins to be expressed in an oblique-gradient fashion (D/T-high pattern), complementary to that of *Ventroptin,* to counteract it (10, 25). The inhibitory effect of FoxG1 on the BMP signaling is thought to be responsible for turning the expression patterns of *BMP2* and *Ventroptin* about 30 degrees to the posterior side from the first D-V axis (10, 25). Switching from *BMP4* to *BMP2* should occur owing to the difference in their genetic regulatory mechanisms, and this would be the basis of the tilting of the D-V axis in the developing retina. The expression of *EphBs*, *ephrin-Bs,* and *ephrin-A2* is under the control of the BMP2 signal (25). BMP2 thus appears to play an important role in the topographic projection along both axes. In this study, we also showed that projections of retinal axons can be manipulated by ectopic expression of these topographic molecules in the retina (25), indicating that topographic retinotectal projection is specified by retinal patterning during development.

(2) Genetic labeling of retinal axons

To save the effort to label retinal axons in mice, we generated transgenic mouse lines in which several sensory systems in the brain are specifically visualized genetically (1). We generated *GAP-lacZ* encoding an axon-targeted reporter, a fusion protein comprising the membrane-anchoring domain of GAP-43 and β-galactosidase. The reporter gene was introduced into the genome under the

control of a promoter element of the *Brn3b* transcription factor to establish transgenic mouse lines. The individual lines thus generated afforded clear images of specific axonal pathways of the visual, vomeronasal, pontocerebellar, and auditory systems. The reporter protein labelled the entire axonal process as well as the cell body of developing and mature neurons on staining with X-gal. We show that these lines facilitate the developmental and anatomical study of these neural systems. This strategy should be applicable to a variety of neural systems by using various specific promoter elements.

(3) Mechanisms for the topographic retinotectal projection

Eph families of RPTK (receptor protein tyrosine kinase) and their ligands, ephrins, play a crucial role in the establishment of retinotectal projections. We identified protein tyrosine phosphatase receptor type O (Ptpro) as a specific PTP that efficiently dephosphorylates both EphA and EphB receptors (22). Ptpro dephosphorylates the second tyrosine residue conserved in the juxtamembrane region (22), which is required for the activation and signal transmission of Eph receptors. Using the chick retinotectal projection system, we showed that Ptpro controls the sensitivity of retinal axons to ephrins and thereby has a crucial role in the establishment of topographic projections (22; **Figure 2**). Our findings explain the molecular mechanism that determines the threshold of the response of RPTKs to the ligands *in vivo*.

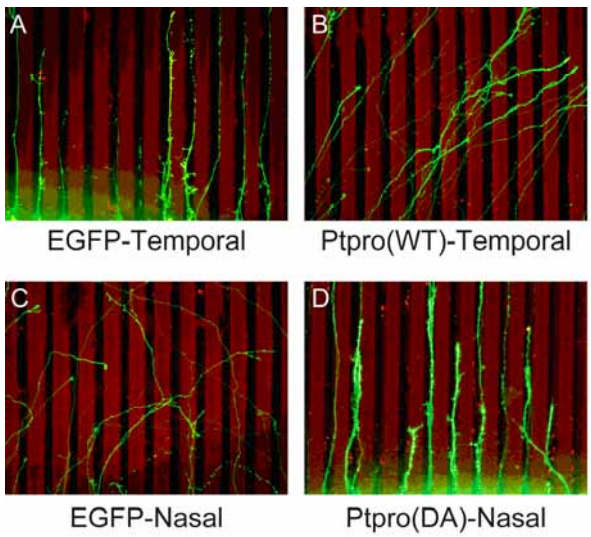

Figure 2. Regulation of the sensitivity of chick retinal axons to ephrin-A2-Fc by Ptpro. To visualize retinal axons, *egfp* was coelectroporated into the retina, and the retinal strips were subjected to the ephrin-A2-Fc stripe assay. (A) Control temporal axons preferred to grow in control Fc-containing lanes (dark) rather than ephrin-A2-Fc lanes (red). (B) Ptpro (WT)-overexpressing temporal axons randomly grew in both control Fc and ephrin-A2-Fc lanes. (C) Control nasal axons randomly grew in both control Fc and ephrin-A2-Fc lanes. (D) When the DA mutant of Ptpro, Ptpro (DA), was overexpressed, nasal axons showed a preference for control Fc lanes like the normal temporal axons.

We identified several region-specific molecules whose expression peaked after E8 in the retina (13): When their expression was experimentally enhanced or suppressed *in vivo*, abnormal axonal targeting, branching, or synaptic arborization was induced. For dynamic morphological changes of cells, rearrangements of the cytoskeleton including microtubules are essential. However, the molecular mechanisms underlying the microtubule remodeling between orientated and disoriented formations are almost unknown. We found that novel subtypes of collapsing response mediator proteins (CRMP-As) in addition to the original proteins (CRMP-Bs), which result from the alternative usage of different first coding exons, are involved in this conversion of microtubules (9). Overexpression of CRMP2A and CRMP2B in chick embryonic fibroblasts induced oriented and disorganized patterns of microtubules, respectively. Moreover, sequential overexpression of another subtype overcame the effect of the former expression of the countersubtype. Overexpression experiments in cultured chick retinas showed that CRMP2B promoted axon branching and suppressed axon elongation in ganglion cells, while CRMP2A blocked these effects when co-overexpressed (9; **Figure 3**). Our findings suggest that the opposing activities of CRMP2A and CRMP2B contribute to the cellular morphogenesis including neuronal axonogenesis through remodeling of the microtubule organization.

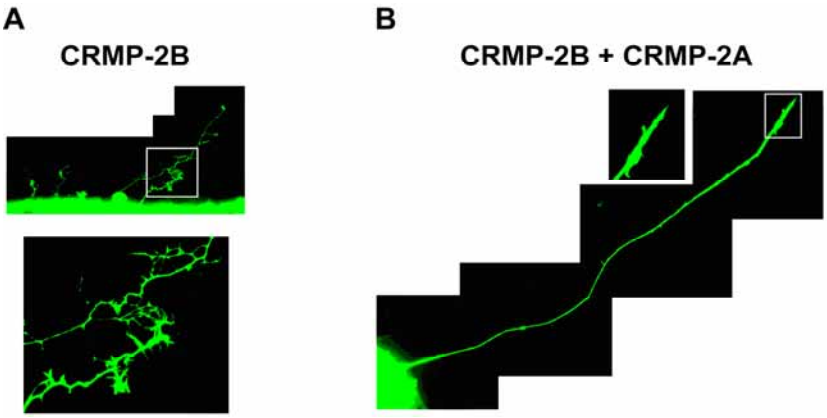

Figure 3. Antagonistic effects of CRMP2A and 2B on axon morphology. Retinas from chick E6 embryos were electroporated with an expression vector to express CRMP2B (A) and CRMP2B + CRMP2A (B), and cultured for 2 days.

We found that another asymmetric molecule, a leucine-rich repeat-containing transmembrane protein (13), associates with adenomatous polyposis coli 2 (APC2), a neuron-specific homologue of the APC tumor suppressor protein. APC2 is distributed along microtubules in growth cones as well as axon shafts of retinal axons (35). Using the chick retinotectal projection system, we showed that APC2 plays an important role in axonal projections through the regulation of cytoskeletal dynamics in response to extracellular signals (35; **Figure 4**): *Apc2* knockdown retinal axons showed abnormal

growth attributable to a reduced response to ephrin-A2 *in vitro*. Overexpression of APC2 in cultured cells induced the stabilization of microtubules, whereas knockdown of *Apc2* in chick retinas with specific short hairpin RNA reduced the stability of microtubules in the retinal axons. Recently, we generated *Apc2*-deficient mice by a gene-targeting technique. We have found defects in the retinocollicular projections of the mutant mice. The mutants also display severe laminary defects in some regions of the brain including the cerebral cortex and cerebellum, indicating that APC2 is involved in axonal navigation and cellular migration (submitted for publication). We are now analyzing the phenotype of the *Apc2*-deficient mouse in more detail to clarify the function of APC2 in the development of the CNS.

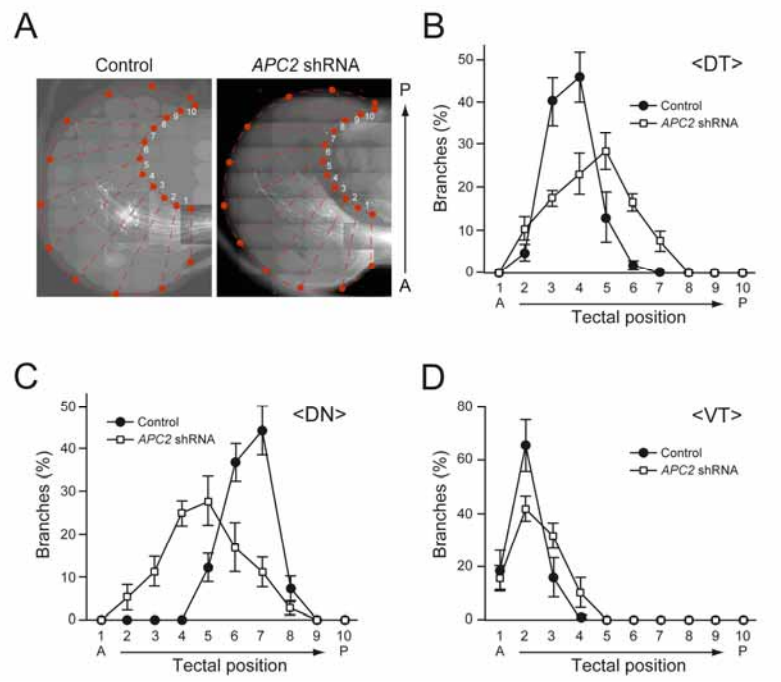

Figure 4. Distribution of interstitial branches of retinal axons on the tectum at E12.5. (A) The tectum was subdivided into 10 areas along the anteroposterior axis, and numbers of branches in each area were counted. (B–D) Quantification of branch distributions of dorsotemporal (DT), dorsonasal (DN), and ventrotemporal (VT) retinal axons, respectively. The values are shown as the mean ± SEM. In APC2-knockdown embryos, interstitial branches from the axon shaft were more diffusely distributed along the anteroposterior axis. A, Anterior; P, posterior.

We expect the molecular mechanisms underlying the branching, pruning, and synapse formation of retinal axons to be revealed through studies on the region-specific molecules in the retina.

(4) 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 a characteristic morphology, physiology, and central projections.

However, our understanding of how these parallel pathways develop is still in its infancy, because few molecular markers corresponding to individual RGC types are available.

We identified a novel secretory protein, SPIG1, expressed predominantly in the dorsal region in the developing chick retina (13). We generated knock-in mice to visualize SPIG1-expressing cells with green fluorescent protein, and found that the mouse retina is subdivided into two distinct domains for SPIG1 expression and SPIG1 effectively marks a unique subtype of RGCs during the neonatal period (28). SPIG1$^+$ cells in the dorsotemporal domain project to the dorsal lateral geniculate nucleus (dLGN), superior colliculus (SC), and accessory optic system (AOS). In contrast, in the remaining region, here named the pan-ventronasal domain, SPIG1$^+$ cells form a regular mosaic and project exclusively to the medial terminal nucleus (MTN) of the AOS that mediates the optokinetic reflex as early as P1 (**Figure 5A;** modified from refs. 28, 33). Their dendrites costratify with ON cholinergic amacrine strata in the inner plexiform layer as early as P3. These findings suggest that these SPIG1$^+$ cells are the ON direction selective (DS) ganglion cells (28). Moreover, a combination of genetic labeling and conventional retrograde labeling revealed that MTN-projecting RGCs are composed of SPIG1$^+$ and SPIG1$^-$ RGCs distributed in distinct mosaic patterns in the retina, indicating that they comprise two functionally distinct subtypes of the ON DS ganglion cells (**Figure 5A**).

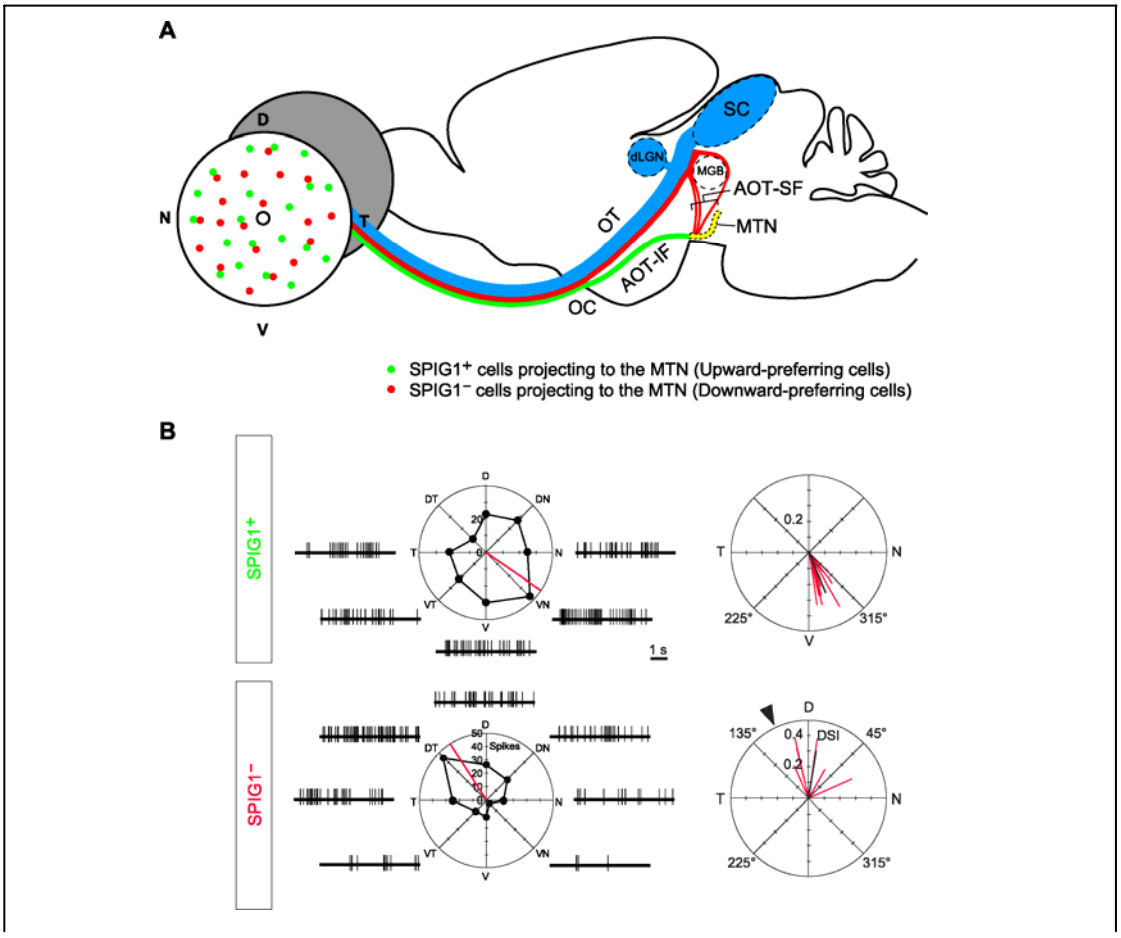

Figure 5. (A) Schematic representation of axonal connectivity between the retina and the contralateral medial terminal nucleus (MTN) of the accessory optic system. Information on upward and downward visual motion is conveyed to the MTN by distinct neuronal pathways. This represents our findings together with those in previous studies on retinal projections to the MTN in mice and rats. The upward-preferring subtype of ON DS ganglion cells ($SPIG1^+$; green) predominantly projects to the MTN via the inferior fasciculus of the accessory optic tract (AOT-IF), whereas the downward preferring subtype ($SPIG1^-$; red) projects to the MTN via the superior fasciculus of the accessory optic tract (AOT-SF). The fibers of the AOT-SF split from the optic tract (OT) and the brachium of the SC, then they course ventrally over the surface of the cerebral peduncle (CP) and finally terminate in the MTN. On the other hand, the fibers of the AOT-IF leave the OT just after passing through the optic chiasm (OC), then course caudally, and terminate in the MTN. MGB, medial geniculate body.
(B) SPIG1 marks the upward-preferring subtype of DS ganglion cells. Representative spike trains and their polar plots in response to eight different directions of the drifting square-wave gratings for 6 s are shown. Spikes were recorded from a $SPIG1^+$ cell (upper panel) and $SPIG1^-$ cell (lower panel). The red line indicates the preferred direction of the cells.

Next, we examined light responses of these two subtypes of MTN-projecting RGCs using targeted electrophysiological recordings (33). $SPIG1^+$ and $SPIG1^-$ RGCs respond preferentially to upward motion and downward motion, respectively, in the visual field (33; **Figure 5B**). The direction selectivity of $SPIG1^+$ RGCs develops normally in dark-reared mice. The MTN neurons are activated by optokinetic stimuli only of the vertical motion as shown by Fos expression analysis. Furthermore, genetic labeling and conventional retrograde labeling revealed that axons of $SPIG1^+$ and $SPIG1^-$ RGCs project to the MTN via different pathways (33; **Figure 5A**). The axon terminals of the two subtypes are organized into discrete clusters in the MTN. These results suggest that information about upward and downward image motion transmitted by distinct ON DS ganglion cells is processed in the MTN separately, if not independently. SPIG1 should therefore serve as a useful molecular marker for studying the differentiation and distinct circuiting of the two ON DS ganglion cell types.

A key circuit module of DS ganglion cells is a spatially asymmetric inhibitory input from starburst amacrine cells in the retina. However, it was not known how and when this asymmetry is established during development. We photostimulated mouse starburst cells targeted with channelrhodopsin-2 (CR-2) while recording from a single $SPIG1^+$ DS ganglion cell. We then followed the spatial distribution of synaptic strengths between starburst and DS ganglion cells during early postnatal development before these neurons can respond to a physiological light stimulus, along with confirmation of connectivity by monosynaptically restricted trans-synaptic rabies viral tracing (41). As a result, we found that random or symmetric synaptic connections from starburst amacrine

cells are established as early as postnatal day 6, and that asymmetric inhibitory synaptic inputs are subsequently developed over a 2-day period (41; **Figure 6**).

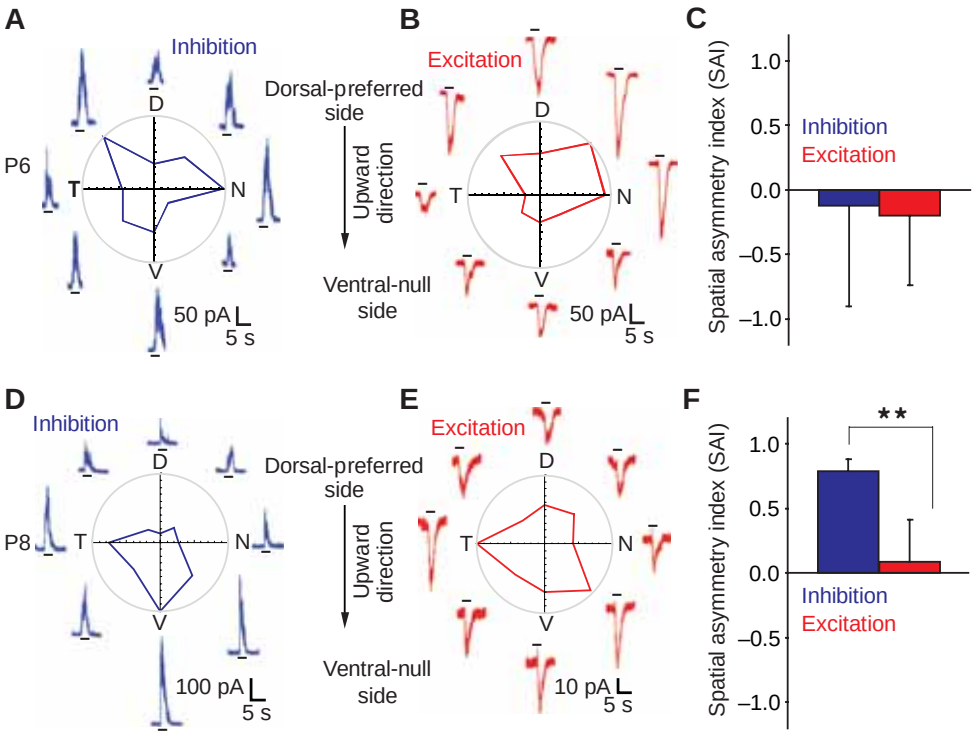

Figure 6. Channelrhodopsin-2-assisted circuit mapping at P6 and P8. Recordings from SPIG1^{+} cells at P6 (A–C) and P8 (D–F). (A–E) Inhibitory (A, D) and excitatory (B, E) postsynaptic currents elicited in a SPIG1^{+} cell by the photostimulation of the surrounding starburst amacrine cells. Polar plots are also shown. (C, F) Spatial asymmetry index (SAI) for inhibition and excitation. Error bars, s.d.

B. Functional roles of protein tyrosine phosphatase receptor type Z

Protein tyrosine phosphorylation plays crucial roles in various biological events including brain development and brain functions. We have made efforts to reveal the functional roles of protein tyrosine phosphatase receptor-type Z, Ptprz (also called PTPζ/RPTPβ), which is mostly expressed as chondroitin sulfate proteoglycans in the brain. To reveal its intracellular signaling pathways, we performed a systematic screening for substrates of Ptprz by developing a "yeast substrate-trapping system", and identified Git1, p190RhoGAP, GOPC/PIST, and Magi1 (17, 1). Subsequent studies revealed that Ptprz selectively dephosphorylates p190RhoGAP at Tyr1105 (ref. 21), Git1 at Tyr-554 (ref. 44), and Magi1 at Tyr-373 and Tyr-858 (ref. 44). Alignment of the primary sequences surrounding the target phosphotyrosine residues showed considerable similarity, suggesting the

presence of a consensus motif for substrate recognition by Ptprz. We estimated the contribution of surrounding individual amino acid residues to the catalytic efficiency using *in vitro* peptide-based assays, and deduced that the typical substrate motif for the catalytic domain of Ptprz was "E/D-E/D-E/D-X-I/V-pY-X" (X is not an acidic residue) (44). We found by database screening that the substrate motif is present in several proteins, including paxillin at Tyr-118. Expectedly, we verified that Ptprz efficiently dephosphorylates paxillin at this site in cells (44).

In the yeast substrate screening above, several PDZ domain-containing proteins including PSD95, Magi3, Veli-3, Synj2bp, Snta1 and Sntb1 were isolated as non-substrate interacting proteins that bind to the C-terminal PDZ-binding motif of Ptprz (17, 1). Among the 21 receptor-type PTP members (RPTPs), only the R5 subfamily, Ptprz and Ptprg, have a canonical PDZ-binding motif (-S-L-V) at their carboxyl-termini. This suggests that Ptprz can form a stable enzyme–substrate complex on PDZ-containing scaffold proteins. Indeed, we found that ErbB4 becomes a substrate for Ptprz with the help of PSD95: In transfected cells, Ptprz can dephosphorylate ErbB4 when PSD95 is coexpressed (27). In addition, TrkA, which is reportedly bound to the PDZ domain of GIPC, was efficiently dephosphorylated by Ptprz (30). Some proteins associated with Ptprz on PDZ-containing scaffold proteins are thus likely dephosphorylated by Ptprz without a strict substrate recognition structure.

The physiological importance of Ptprz has been demonstrated through studies with *Ptprz*-deficient mice (Neurosci. Lett. *247*, 135-138, 1998). Although homozygous *Ptprz*-deficient mice appeared to be healthy and showed no obvious anatomical abnormalities in the brain, we found that one conspicuous defect in adult *Ptprz*-deficient mice is a significant enhancement of long term potentiation (LTP) in the CA1 region of the hippocampus (18) and learning deficits (18, 21). LTP is a long-lasting augmentation of synaptic strength that has been suggested as a cellular mechanism underlying learning and memory. Because Ptprz is known to be abundant in the synaptic fraction, it is considered to be involved in the regulation of synaptic plasticity, and learning and memory. In this regard, the phosphorylation of p190RhoGAP at Tyr-1105, was found to be decreased after fear conditioning in the hippocampus of wild-type mice, but not of *Ptprz*-deficient mice (21), suggesting that the PTP activity of Ptprz is up-regulated during learning.

We had already identified pleiotrophin/HB-GAM and midkine as ligands involved in the regulation of Ptprz (J.B.C. *271*, 21446-21452, 1996; J.B.C. *274*, 12474-12479, 1999; Proc. Natl. Acad. Sci. USA *97*, 2603-2608, 2000). We revealed that binding of pleiotrophin to the extracellular region of Ptprz induces dimerization (or oligomerization) and thereby suppresses the PTP activity (23). In addition, we found that the extracellular region is proteolytically processed *in vivo* by several proteases including metalloproteinases (31) or plasmin (32). Since matrix metalloproteinase 9 (MMP-9) levels and accordingly the proteolytic activity are rapidly increased by stimuli that induce LTP, the removal of the inhibitory extracellular portion by metalloproteinase-mediated processing presumably abolishes

this ligand-induced inactivation mechanism (**Figure 7A, B**), and thereby induces dephosphorylation of the substrate proteins in the hippocampus of wild-type mice.

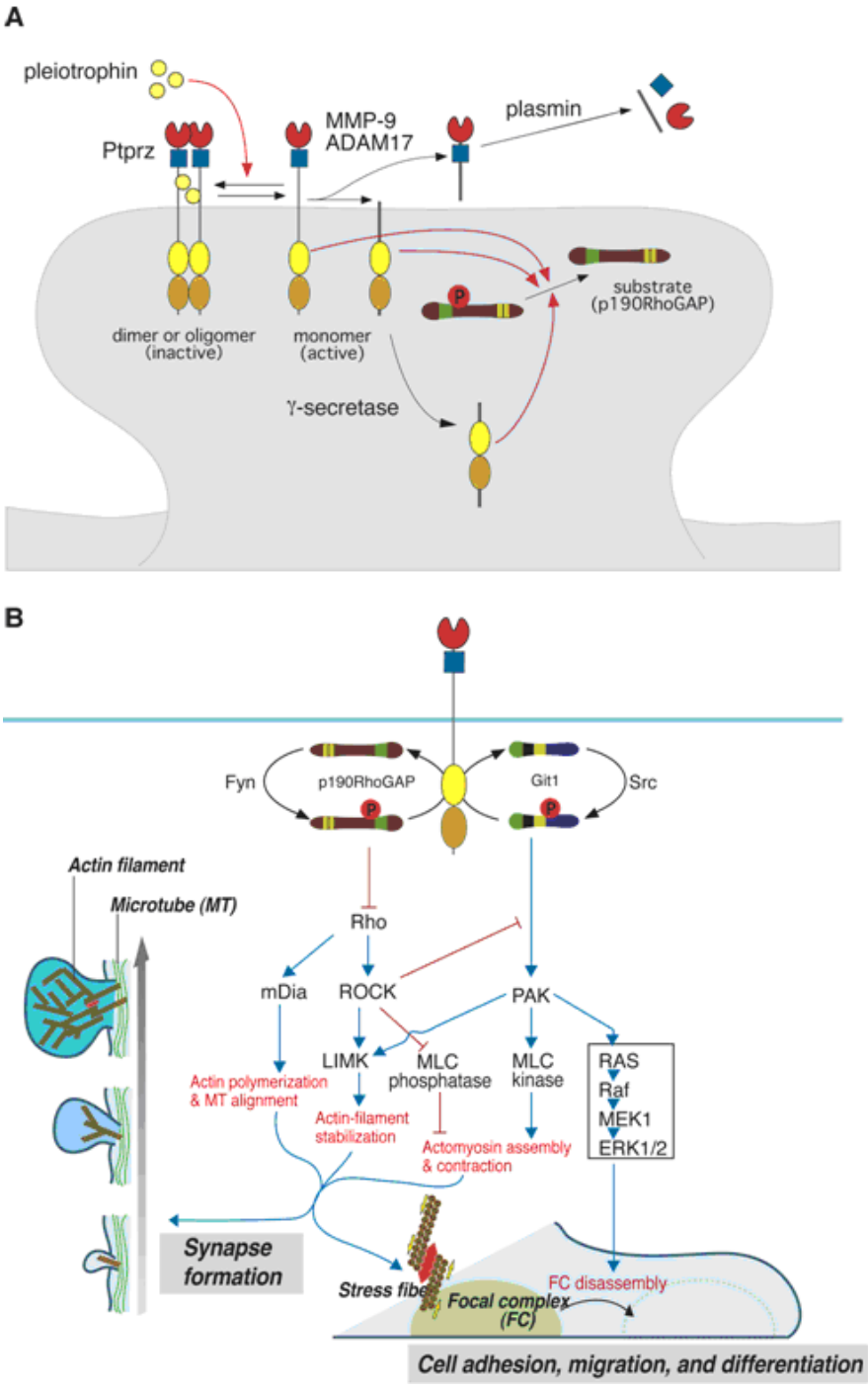

Figure 7. (A) Schematic drawing of the proteolytic processing of Ptprz in central synapses.
(B) Schematic drawing of proposed Ptprz signaling pathways for the regulation of synaptic plasticity (Spine formation) and the induction of gastric ulcers (Cell adhesion of epithelial cells).

In addition to the brain, we found that the non-proteoglycan form of Ptprz-B, a splicing isoform of Ptprz, is expressed in the gastric epithelial cells. Here, we demonstrated that Ptprz functions as a receptor for the vacuolating cytotoxin VacA, produced by *Helicobacter pylori* (5): Surprisingly, *Ptprz*-deficient mice did not show mucosal damage at all when VacA was administered intragastrically, although VacA was incorporated into the gastric epithelial cells to the same extent as in wild-type mice (5). VacA bound to Ptprz, and the levels of tyrosine phosphorylation of Git1, a Ptprz substrate, were higher after treatment with VacA, indicating that VacA behaves as a ligand for Ptprz (5). Thus, erroneous Ptprz signaling by VacA induces gastric ulceration (**Figure 7B**).

Our understanding of the physiological functions of Ptprz, together with its intracellular signaling mechanisms through substrates, has become much deeper over the past decade. We are now focusing our efforts on integrating these findings into a comprehensive picture. Furthermore, we are planning to expand this strategy to other RPTPs to know their functional roles.

C. Mechanisms of homeostatic control of body fluids in the mammalian brain

Mammals have a set of homeostatic mechanisms that work together to maintain body-fluid sodium (Na^+) levels at 135–145 mM and osmolality at ~300 mOsm/kg through the control of intake and excretion of salt and water. The circumventricular organs (CVOs), where no blood-brain barrier exists, are suggested to be involved in monitoring body-fluid conditions. However, molecular and cellular mechanisms for sensing the Na^+ levels and osmolality of body fluids within the brain have long been an enigma. We have been investigating the mechanisms of homeostatic control of body fluids in the mammalian brain, including Na^+-level- and osmolality-sensing systems.

As for Na^+-level-sensing, our series of studies with *Na*$_x$-deficient mice revealed that Na_x serves as a Na^+-level sensor of body fluids (2, 3, 8, 15, 19; 2, 3). Na_x, an atypical sodium channel whose structure is poorly homologous (~50%) to the voltage-gated sodium channels, had long remained an enigma (**Figure 8A**). We generated *Na*$_x$-gene deficient mice by inserting the *lacZ* gene in-frame and analyzed the expression pattern of Na_x. In the central nervous system, *Na*$_x$ was expressed in the dorsal root ganglions and the CVOs including the subfornical organ (SFO), organum vasculosum of the lamina terminalis (OVLT), median eminence (ME), and posterior pituitary (PP) (**Figure 8B**; see also J. Neurosci. *20*, 7743-7751, 2000). In the peripheral nervous system, Na_x was expressed in the non-myelinating Schwann cells (3). *Na*$_x$-deficient mice did not stop ingesting salt when dehydrated, while wild-type mice avoided salt (J. Neurosci. *20*, 7743-7751, 2000). We subsequently demonstrated that the Na_x channel is a concentration-sensitive Na channel with a threshold value of ~150 mM for extracellular Na^+ *in vitro* (2). We further showed that salt aversive behavior does not occur on direct infusion of a hypertonic Na^+ solution into the cerebral ventricle in *Na*$_x$-deficient mice,

in contrast to wild-type mice (15). The behavioral defect of *Na$_x$*-deficient mice was completely recovered by a site-directed transfer of the *Na$_x$* gene into the SFO, indicating that the SFO is the primary site of the Na^+-level-sensing for the control of salt-intake behavior (15). All these findings indicate that the Na_x channel is the brain Na^+-level sensor, which has been long postulated to be present at the sensory CVOs (2, 3).

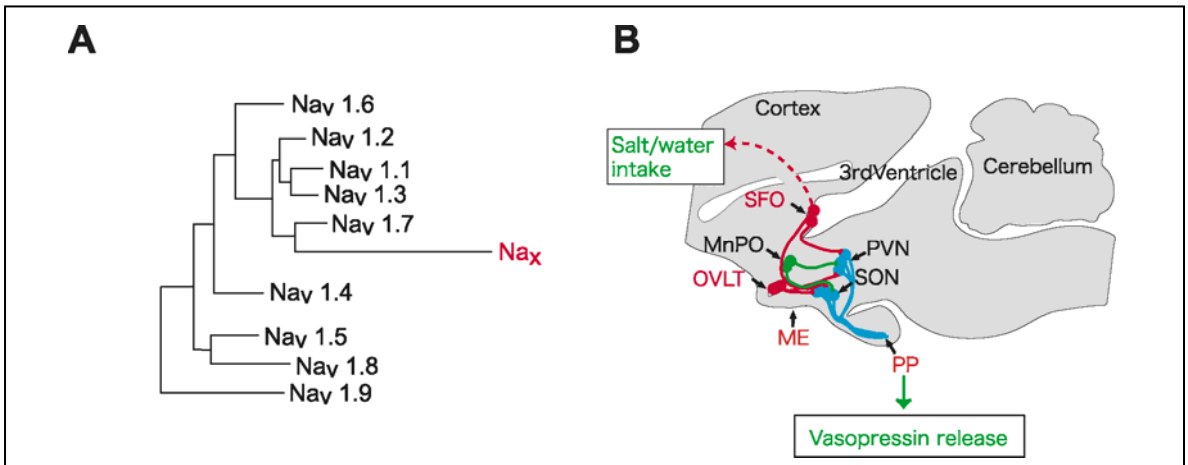

Figure 8. (A) Phylogenetic tree of voltage-gated sodium channel α subunits. (B) Schematic illustration of the midsagittal section of the mouse brain. Nuclei (filled circles) and neural pathways (lines) involved in the regulation of vasopressin release are shown. The neural pathway involved in salt/water intake remains unknown (dotted line). The sensory CVOs, including the SFO, are characterized by the presence of neuronal cell bodies and extensive networks of fenestrated capillaries which allow components of plasma to be released into the intercellular space. The ventricular side is partitioned by an ependymal cell layer facing the third ventricle. Na_x is expressed in the SFO, OVLT, ME, and PP. MnPO, median preoptic nucleus; PVN, paraventricular nucleus; SON, supraoptic nucleus.

Our detailed analyses revealed that Na_x channels in the SFO are specifically expressed in perineuronal processes of astrocytes and ependymal cells enveloping particular neural populations including GABAergic neurons (20). This indicates that glial cells, not neurons, are the primary site of Na^+-level-sensing, suggesting some mechanism to transfer the Na^+ signals from "inexcitable" glial cells to neurons. We subsequently demonstrated that Na_x channels directly interact with α subunits of Na^+/K^+-ATPase, and thereby bring about Na^+-dependent activation of the metabolic state of the glial cells (26). Metabolic enhancement leading to the extensive production of lactate was observed under high Na^+ conditions in the SFO of wild-type mice, but not of *Na$_x$*-deficient mice (26). Lactate, as well as Na^+, stimulated the firing activity of GABAergic neurons in the SFO (26). The GABAergic neurons supposedly regulate hypothetic projecting neurons involved in the control of salt-intake behavior (26; **Figure 9**). To our knowledge, this study is the first to show that glial cells take the

initiative in the regulation of neural activity using lactate as a signaling substance, a “gliotransmitter”.

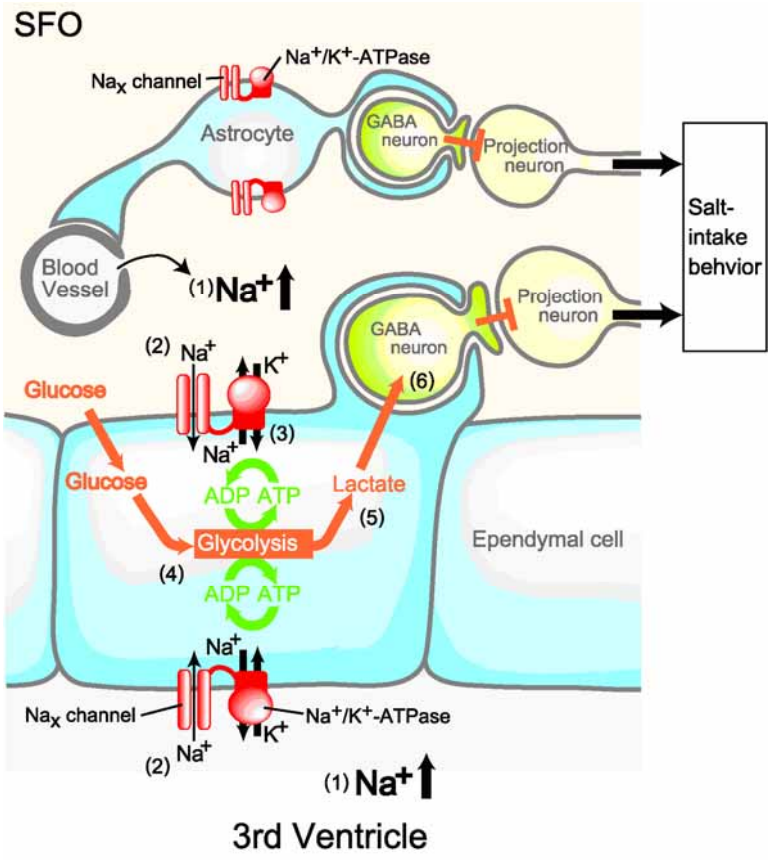

Figure 9. Schematic overview of the Na^+-level-sensing mechanism and Na^+-dependent regulation of neurons in the SFO. (1) When animals are dehydrated, Na^+ concentrations in plasma and cerebrospinal fluid increase above the normal level of ~145 mM. (2) When the extracellular Na^+ concentration exceeds ~150 mM, Na_x channels begin to open, and the intracellular Na^+ concentration in these glial cells is increased. (3) This leads to the activation of Na^+/K^+-ATPase. The activated Na^+/K^+-ATPase consumes more ATP than usual to pump out Na^+. (4) To fuel Na^+/K^+-ATPase with ATP, the glial cells enhance glucose uptake to stimulate anaerobic glycolysis. (5) Lactate, the end product of anaerobic glycolysis, is released from the glial cells and supplied to neurons, including GABAergic neurons, through the processes enveloping them. (6) Lactate stimulates the activity of the GABAergic neurons through production of ATP, leading to the inhibition of hypothetic projecting neurons involved in the control of salt-intake behavior. In *Na*$_x$-deficient mice, the Na^+-dependent stimulation of glycolysis is impaired and the activity of the GABAergic neurons is not promoted even under dehydrated conditions, and so the salt-intake behavior is not suppressed.

Recently, we found a patient with essential hypernatremia who harbors autoantibodies to Na_x (38). The patient had a ganglioneuroma mainly composed of Na_x-positive Schwann-like cells, and this neoplasia likely evoked an antitumor immune response (38). Passive transfer of the

immunoglobulin (Ig) fraction of the patient's serum reproduced the symptoms with abnormal reductions in water intake and vasopressin release in wild-type mice (38; **Figure 10**). Because *Na_x*-deficient mice are normal in vasopressin release (37), we considered that autoantibodies to Na_x not only affected the Na^+-level-sensing mechanisms mediated by Na_x channels, but also impaired the regulatory systems for the production/release of vasopressin. In support of this view, cell death was observed in the brain of mice injected with the patient's Ig, along with focal deposits of complement C3 and inflammatory infiltrates in CVOs. This suggests that complement-mediated cell death in the CVOs occurred in these mice and also in the patient (38). Because the SFO and OVLT have efferent projections, which are responsible for the regulation of vasopressin, histological damage to the SFO and OVLT would be the reason for the dysregulation of vasopressin production/release. In addition, damage to the PP, the site where vasopressin is released into the blood circulatory system, would also affect the release. These results suggest that the ganglioneuroma triggered the production of anti-Na_x autoantibodies, leading to persistent tissue damage in the CVOs, and thereby hypernatremia (a paraneoplastic neurologic disorder).

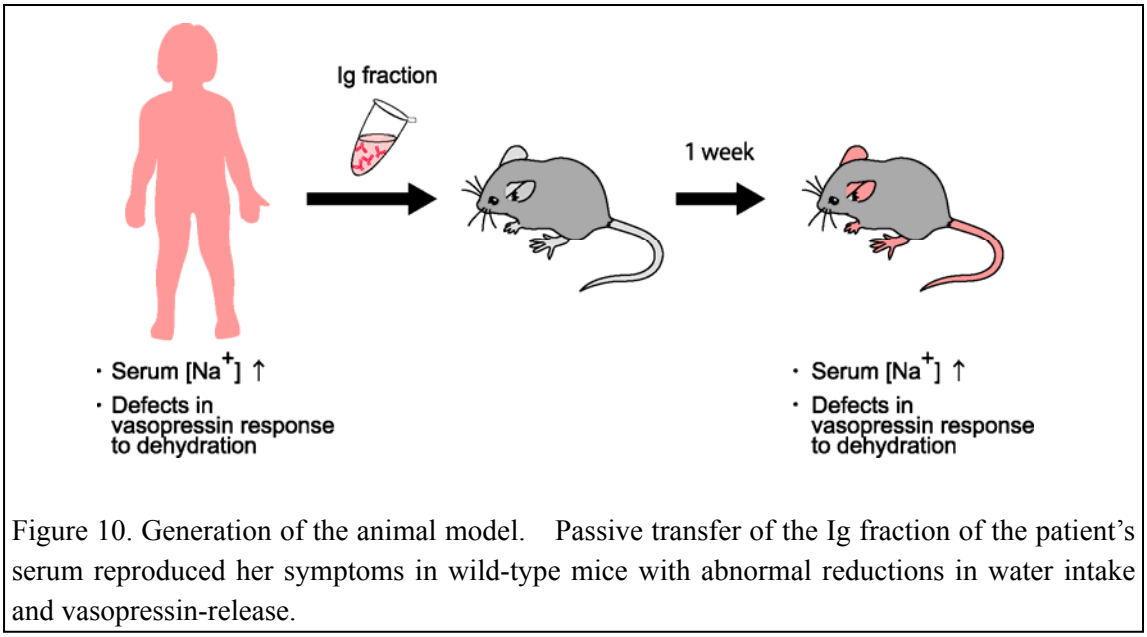

Figure 10. Generation of the animal model. Passive transfer of the Ig fraction of the patient's serum reproduced her symptoms in wild-type mice with abnormal reductions in water intake and vasopressin-release.

As for the osmolality-sensing mechanism, a cation channel, transient receptor potential vanilloid 1 (TRPV1), has been implicated as a sensor of hypertonicity in body fluids based on studies with *TRPV1*-deficient mice. However, the response of TRPV1 to hypertonic stimuli has not been demonstrated with heterologous expression systems so far, despite intense efforts by several groups. Thus, the molecular entity of the hypertonic sensor *in vivo* still remains controversial. Very recently, we found that the full-length form of TRPV1 is sensitive to an osmotic increase exclusively at around body temperature (~37°C) using a heterologous expression system (43). We also demonstrated that

the osmosensitivity of TRPV1 at 36°C is further enhanced by another activating stimulus, such as protons (pH) or capsaicin, indicating that osmosensitivity of TRPV 1 is synergistically enhanced by these distinct activating stimuli. Our findings thus indicate that TRPV1 integrates multiple types of activating stimuli, and is sensitive to hypertonic stimuli under physiologically relevant conditions.

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