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Nav1.2 is expressed in caudal ganglionic eminence-derived disinhibitory interneurons: Mutually exclusive distributions of Nav1.1 and Nav1.2

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ABSTRACT

Nav1.1 and Nav1.2 are the voltage-gated sodium channel pore-forming alpha I and II subunits, encoded by the genes *SCN1A* and *SCN2A*. Although mutations of both genes have similarly been described in patients with epilepsy, autism and/or intellectual disability, their expression sites in brain are largely distinct. Nav1.1 was shown to be expressed dominantly in parvalbumin (PV)-positive or somatostatin (SST)-positive inhibitory neurons and in a sparsely-distributed subpopulation of excitatory neurons. In contrast, Nav1.2 has been reported to be dominantly expressed in excitatory neurons. Here we show that Nav1.2 is also expressed in caudal ganglionic eminence (CGE)-derived inhibitory neurons, and expressions of Nav1.1 and Nav1.2 are mutually-exclusive in many of brain regions including neocortex, hippocampus, cerebellum, striatum and globus pallidus. In neocortex at postnatal day 15, in addition to the expression in excitatory neurons we show that Nav1.2 is expressed in reelin (RLN)-positive/SST-negative inhibitory neurons that are presumably single-bouquet cells because of their cortical layer I-limited distribution, and vasoactive intestinal peptide (VIP)-positive neurons that would be multipolar cell because of their layer I/II margin and layer VI distribution. Although Nav1.2 has previously been reported to be expressed in SST-positive cells, we here show that Nav1.2 is not expressed in either of PV-positive or SST-positive inhibitory neurons. PV-positive and SST-positive inhibitory neurons derive from medial ganglionic eminence (MGE) and innervate excitatory neurons, while VIP-positive and RLN-positive/SST-negative inhibitory neurons derive from CGE, innervate on inhibitory neurons and play disinhibitory roles in the neural network. Our results therefore indicate that, while Nav1.1 is expressed in MEG-derived inhibitory neurons, Nav1.2 is expressed in CGE-derived disinhibitory interneurons in addition to excitatory neurons. These findings should contribute to understanding of the pathology of neurodevelopmental diseases caused by *SCN2A* mutations.

1. Introduction

Voltage-gated sodium channels are transmembrane glycoprotein complexes that play a critical role in generation and propagation of action potentials in excitable cells including neurons, and consist of one pore-forming alpha-subunit and one or two accessory beta-subunits. In mammalian brain, four α subunits, namely, Nav1.1, 1.2, 1.3, and 1.6 encoded by *SCN1A*, *2A*, *3A*, and *8A*, respectively, are heterogeneously expressed mainly at axon initial segments (AISs) and nodes of Ranvier. Although some previous studies proposed dominant somatodendritic distribution of Nav1.1 in excitatory neurons [1–3], we and others revealed that axons of PV- or SST-positive inhibitory interneurons in neocortex and hippocampus heavily express Nav1.1 [4–9]. We also showed that Nav1.1 is sparsely expressed in axons of a distinct subpopulation of neocortical layer V excitatory neurons [6]. In contrast, dominant Nav1.2 expression in excitatory neurons has been widely accepted. In neocortex, Nav1.2 is expressed at proximal AISs and nodes of

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Ranvier of pyramidal cells [9,10]. In hippocampus, Nav1.2 is densely observed in unmyelinated mossy fibers of hippocampal granule cells [1,3] and in axons of CA1 and CA3 pyramidal cells [3,11]. In cerebellum, parallel fibers of granule cells densely express Nav1.2 [1,3,12]. Nav1.2 has been reported to be detected in GABAergic neurons such as striatal medial spiny neurons [13] and neocortical SST-positive interneurons [7], though the latter reveals to be contradictory to our present study.

Here, we report mutually-exclusive expressions of Nav.1.1 and Nav1.2 in multiple brain regions such as neocortex, hippocampus, cerebellum, striatum and globus pallidus. Moreover, in neocortex and hippocampus we show that Nav1.2 is expressed in CGE-derived GABAergic interneurons such as RLN-positive/SST-negative single-bouquet cells and VIP-positive multipolar cells. We do not observe Nav1.2 in either of PV-positive or SST-positive neurons, both are MGE-derived GABAergic interneurons.

2. Materials and methods

2.1. Animals

GAD67-GFP knock-in and *Vgat*-Venus transgenic mice expressing a fluorescent protein specifically in inhibitory cells were described previously [14,15], and maintained on a C57BL/6 J background. All animal experimental protocols were approved by the Animal Experiment Committee of RIKEN Institute.

2.2. Tissue preparation and immunohistochemistry

Mouse tissues were fixed using periodate-lysine-4% paraformaldehyde (PLP), postfixed in PLP for overnight at 4 °C, embedded in paraffin, and cut in 6 μm sections. The sections were deparaffinized, rehydrated, microwaved in 1 mM EDTA, pH8.0, and blocked in phosphate-buffered saline (PBS) containing 0.05% Tween 20, 4% BlockAce (Dainippon Sumitomo Pharma, Osaka, Japan) and endogenous avidin and biotin blocker (Avidin/Biotin or Streptavidin/Biotin Blocking Kit: Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. The sections were then incubated with the goat anti-internal-region Nav1.2 antibody (1:500; SC-31371, G-20, Santa Cruz Biotechnology, Santa Cruz, CA, USA), the rabbit anti-internal-region Nav1.2 (1:500; ASC-002, Alomone, Jerusalem, Israel) or the goat anti-Nav1.1 antibody (1:500; SC-16031, C-18, Santa Cruz Biotechnology) for 12–15 h at 4 °C. Endogenous peroxidases were quenched by incubation with 0.3% hydrogen peroxide in PBS. The sections were further incubated with biotinylated goat polyclonal secondary antibody (1:200; BA-9500, Vector Laboratories). Detection of antibody–antigen complexes was accomplished using the Vectastain Elite ABC kit (PK-6100, Vector Laboratories) and the NovaRed substrate kit (SK-4800, Vector Laboratories). For the double staining, the sections, in which the antibody-antigen complexes were visualized with the NovaRed substrate (Vector Laboratories), were subsequently incubated with the mouse anti-GFP antibodies (1:500; 11814460001, clones 7.1 and 13.1, Roche Diagnostics, Indianapolis, IN, USA), the mouse anti-reelin antibody (1:1000; ab78540, G-10, Abcam, Cambridge, United Kingdom), the rabbit anti-vasoactive intestinal peptide antibody (1:1000; 20077, ImmunoStar, Inc., Hudson,WI, USA), the rabbit anti-somatostatin antibody (1:1000; T-4103, Peninsula Laboratories, San Carlos, CA, USA) or the goat anti-Nav1.1 antibody (1:500; C-18, Santa Cruz Biotechnology). Detection of the second antibody–antigen complexes was accomplished using the Vectastain ABC-AP kit (AK-5000, Vector Laboratories) and the alkaline phosphatase substrate kit III (SK-5300, Vector Laboratories).

For immunofluorescence histochemistry, the sections were pretreated with Lambda Protein Phosphatase (P0753S, New England Biolabs, Ipswich, MA, USA) to enhance the binding of the rabbit anti-Nav1.2 (ASC-002, Alomone) to Nav1.2 by removing the phosphate group on the antigen. The sections were then incubated with the rabbit anti-Nav1.2 (1:500; ASC-002, Alomone), the mouse anti-ankyrinG (1:250; SC-12719, Santa Cruz Biotechnology), and the goat anti-Nav1.1 antibody (1:500, C-18, Santa Cruz Biotechnology), and incubated with the secondary antibodies Alexa Flour 594, 647 (1:1000; Thermo Fisher Scientific, Waltham, MA USA) and Biotin conjugated (1:200; Vector Laboratories). Biotinylated anti-rabbit IgG antibody was detected using the Streptavidin conjugated Alexa Flour 488 (Thermo Fisher Scientific). Sections were mounted with Antifade Vectashield mounting medium containing 4′-6-diamidino-2-phenylindole (DAPI) (Vector Laboratories) to stain nuclei. Images were captured using Biozero BZ-8100, BZX-710 microscope (Keyence, Osaka, Japan) and TCS SP2 microscope (Leica Microsystems, Wetzler, Germany), and processed using Adobe Photoshop Elements 10 (Adobe Systems, San Jose, CA, USA).

3. Results

3.1. Nav1.2 and Nav1.1 are distributed in mutually-excusive manner

We immunohistochemically investigated expression sites of Nav1.2 and Nav1.1. Because both Nav1.1 and Nav1.2 immunosignals at AISs have been most dense at around postnatal day (P) 15 during development in previous studies [6,11], we focused on P14.5-P15.5. At first, we performed single labeling of Nav1.2 or Nav1.1 by using the *Vgat*-Venus transgenic mice, which expressed GFP-derived fluorescent protein Venus in global inhibitory neurons [15]. In neocortex, Nav1.2-immunoreactivity was pronounced in most axons of Venus−negative excitatory neurons such as neocortical pyramidal cells (Fig. 1A and B). While Nav1.2-imunoreactivity was indiscernible in AISs of most Venus-positive neurons, it was sparsely observed in a minor population of Venus-positive cells such as those in neocortical layer I (Fig. 1B). Meanwhile, Nav1.1-immunoreactivity was dense at AISs of a major subpopulation of GABAergic neurons (Fig. 1D and E), which are assumed to be PV-positive basket [4,6] or SST-positive Martinotti [7,8] inhibitory neurons, though we previously showed that a distinct subpopulation of excitatory neurons are Nav1.1-positive [6]. In hippocampus, Nav1.2-immunoractivity was apparent in most axons of excitatory neurons such as hippocampal CA pyramidal and dentate granule cells (Fig. 1A,C, Supplementary Figs. 1A–C). Venus-negative cells in hilus, presumably mossy cells that are excitatory neurons, were also Nav1.2-positive (Supplementary Fig. 1C). Nav1.2-immunoreactivity was not observed in AISs of most Venus-positive neurons, but observed in a minor population of Venus-positive neurons in lacnosum-moleculare/deep radiatum. Meanwhile, Nav1.1-imunoreactivity was observed at AISs of a major subpopulation of GABAergic neurons (Fig. 1D, F and Supplementary Figs. 1E–G), which are assumed to be PV-positive basket [4,6] or SST-positive HIPP or O-LM [7,8] inhibitory neurons, while it was not observed in excitatory neurons. In cerebellum, Nav1.2-immmunoreactivity was observed in parallel fibers of granule cells which are excitatory neurons (Supplementary Fig. 1D). As we showed previously [6], Nav1.1-immunoreactivity was apparent in axons of basket cells, but here we newly found that Venus-positive neurons in granule cell layer, presumably Golgi cells that are GABAergic cells, are also Nav1.1-positive (Supplementary Fig. 1H). In our previously study, we showed Nav1.1 is expressed in Purkinje cells [6] which are again GABAergic neurons.

Double labeling (Fig. 2) further confirmed that Nav1.2 and Nav1.1-immunosignals were distributed in a mutually-exclusive manner in neocortex (Fig. 2B and H), hippocampus (Fig. 2C, D and I) and other brain regions including cerebellum (Fig. 2E),