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Nav1.1 haploinsufficiency in excitatory neurons ameliorates seizure-associated sudden death in a mouse model of Dravet syndrome

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Dravet syndrome is a severe epileptic encephalopathy mainly caused by heterozygous mutations in the SCN1A gene encoding a voltage-gated sodium channel Nav1.1. We previously reported dense localization of Nav1.1 in parvalbumin (PV)-positive inhibitory interneurons in mice and abnormal firing of those neurons in Nav1.1-deficient mice. In the present study, we investigated the physiologic consequence of selective Nav1.1 deletion in mouse global inhibitory neurons, forebrain excitatory neurons or PV cells, using vesicular GABA transporter (VGAT)-Cre, empty spicules homolog 1 (Emx1)-Cre or PV-Cre recombinase drivers. We show that selective Nav1.1 deletion using VGAT-Cre causes epileptic seizures and premature death that are unexpectedly more severe than those observed in constitutive Nav1.1-deficient mice. Nav1.1 deletion using Emx1-Cre does not cause any noticeable abnormalities in mice; however, the severe lethality observed with VGAT-Cre-driven Nav1.1 deletion is rescued by additional Nav1.1 deletion using Emx1-Cre. In addition to predominant expression in PV interneurons, we detected Nav1.1 in subpopulations of excitatory neurons, including entorhino-hippocampal projection neurons, a subpopulation of neocortical layer V excitatory neurons, and thalamo-cortical projection neurons. We further show that even minimal selective Nav1.1 deletion, using PV-Cre, is sufficient to cause spontaneous epileptic seizures and ataxia in mice. Overall, our results indicate that functional impairment of PV inhibitory neurons with Nav1.1 haploinsufficiency contributes to the epileptic pathology of Dravet syndrome, and show for the first time that Nav1.1 haploinsufficiency in excitatory neurons has an ameliorating effect on the pathology.

INTRODUCTION

Heterozygous mutations in the SCN1A gene, which encodes a voltage-gated sodium channel α1 subunit (Nav1.1), are associated with a wide spectrum of childhood epilepsies, including generalized epilepsy with febrile seizures [Mendelian Inheritance in Man (MIM) #604233], and Dravet syndrome (MIM #607208) (1–8). Dravet syndrome is an intractable epileptic encephalopathy...
characterized by early onset epileptic seizures, mental retardation, autistic behaviors, ataxia and increased risk of sudden unexpected death in epilepsy (SUDEP) (9,10). Nav1.1 haploinsufficiency has been implicated in Dravet syndrome pathology (11–13) and in mice, causes epileptic seizures, ataxia (14,15), learning/memory impairments and abnormal social behaviors (16,17). Previously, we showed dense Nav1.1 expression at the axons and soma of parvalbumin (PV)-positive interneurons, a subclass of GABAergic inhibitory neurons, in the hippocampus and neocortex. In Scn1a heterozygous knock-out mice, these cells exhibit pronounced spike amplitude decrements in trains of evoked action potentials. Therefore, based on these observations, we proposed that impaired functions of PV interneurons arising from Nav1.1 haploinsufficiency contribute to the generation of seizures in patients with Dravet syndrome (15). A recent study confirmed predominant Nav1.1 expression in PV interneurons (18).

Cheah et al. (19) recently reported spontaneous epileptic seizures in mice with a conditional Nav1.1 deletion mediated by the distal-less homeobox (Dlx)1/2/11b enhancer + β-globin promoter Cre driver (Dlx1/2-Cre) (19), which express Cre recombinase selectively in all forebrain GABAergic inhibitory neurons (20). Subsequently, Dutton et al. (18) reported increased flurothyl- and hyperthermia-induced seizure susceptibilities in mice with conditional Nav1.1 inactivation, mediated by the protein phosphatase 1 regulatory subunit 2 (Ppp1r2) promoter-driven Cre driver (Ppp1r2-Cre) (18), which express Cre recombinase in a subset of forebrain GABAergic neurons mainly consisting of PV interneurons, but also including reelin- and neuropeptide Y-positive inhibitory neurons (21). Dutton et al. (18) also showed that Emx1-Cre-dependent Nav1.1 inactivation did not alter flurothyl- and hyperthermia-induced seizure susceptibilities in mice. These studies provide important insights into the functional roles of neuronal subtypes in the epileptic pathology of Dravet syndrome. However, much remains to be investigated, including the effect on the epileptic pathology, of Nav1.1 deletion in PV cells.

Here, we report that selective Nav1.1 deletion in global inhibitory neurons, via VGAT-Cre recombination, causes severe epileptic seizures and sudden death in mice. Although previous studies show that Ppp1r2-Cre or Dlx1/2-Cre-driven elimination of Nav1.1 leads to comparable or even milder phenotypes, compared with constitutive Scn1a knock-out mice (18,19), our VGAT-Cre-driven elimination of Nav1.1 in global inhibitory neurons results in a more severe phenotype than constitutive Scn1a knock-out mice. Furthermore, we show that additional Nav1.1 deletion in mouse excitatory neurons, using Emx1-Cre recombination, does not cause any noticeable abnormalities on its own, but improves the severe phenotype observed in mice with VGAT-Cre-dependent Nav1.1 deletion. In addition, we show that a minimal amount of PV-Cre-driven Nav1.1 deletion is sufficient to cause spontaneous epileptic seizures and ataxia in mice. These results indicate that Nav1.1 haploinsufficiency in excitatory neurons has an ameliorating effect in Dravet syndrome, and support our previous proposal that functional impairment of PV interneurons is the cellular/circuit basis of epileptic pathology in Dravet syndrome (15).

RESULTS

Generation of mice with a floxed Scn1a allele

We generated mice with a floxed Scn1a allele, containing two loxP cassettes placed on either side of coding exon 7 (Fig. 1A and Supplementary Material, Fig. S1). Mice homozygous for the floxed allele (Scn1a<sup>d/d</sup>) were viable with no obvious abnormal phenotypes, and expressed normal Nav1.1 levels in the brain (Fig. 1B). Next, we generated mice with a constitutively deleted Scn1a allele by crossing Scn1a<sup>d/d</sup> mice and Ella-Cre mice, in which the Cre-loxP recombination occurs in germline cells (Fig. 1A and C) (22). Homozygous (Scn1a<sup>d/d</sup>) progeny were viable with no obvious abnormal phenotypes in the first postnatal week. However, the mice developed severe ataxia around postnatal day 10 (P10) and generalized convulsive seizures around P12 (Fig. 1D and E). Scn1a<sup>d/d</sup> mice also developed malnutrition in the third postnatal week, probably due to ataxia and recurrent seizures, and died before P20 with an average lifespan of 16.2 days (n = 19) (Fig. 1F). Heterozygous (Scn1a<sup>d/+</sup>) progeny were viable with no obvious abnormal phenotypes until the beginning of the third postnatal week. By the end of the third postnatal week, the mice developed recurrent seizures. Some Scn1a<sup>d/+</sup> mice suffered sporadic sudden death after P18, and 25.0% (9 of 36) died before P90 (Fig. 1F). These clinical manifestations of Scn1a<sup>d/d</sup> and Scn1a<sup>d/+</sup> mice are virtually identical to those previously reported in Nav1.1-null mice (14,15), indicating that mice carrying the floxed Scn1a allele can serve as useful tools for the dissection of the physiologic consequences of Nav1.1 deletion, in genetically defined neuronal subtypes, using the Cre-loxP system.

Selective Nav1.1 deletion in global inhibitory neurons in mice causes epileptic seizures with higher lethality than constitutive knock-outs

To assess the physiologic consequences of Nav1.1 deletion in global GABAergic neurons, we generated a BAC transgenic mouse line with Cre recombinase expression under the control of the promoter for vesicular GABA transporter (VGAT) that is expressed in glycinergic and GABAergic inhibitory neurons (23,24) (Fig. 2A). The Vgat-Cre BAC transgenic (VGAT-Cre) mice were viable with no obvious abnormal phenotypes. Immunohistochemical investigation of VGAT-Cre, Rosa26-LacZ double-mutant mice (25) revealed that transgenic Vgat promoter-driven Cre-loxP recombination occurred widely in brain tissue, including the olfactory bulb, striatum, neocortex, hippocampus, cerebellum, medulla and pons (Fig. 2B). Immunofluorescent examination showed β-galactosidase immunosignals present in 96.5% (1816 of 1882) and 93.8% (422 of 450) of GABA-positive cells in the hippocampus and neocortex, respectively (Fig. 2C–J), suggesting transgenic Vgat promoter-driven Cre-loxP recombination occurred globally in whole populations of GABAergic neurons. Conversely, 99.0% (1816 of 1835) and 88.5% (422 of 477) of β-galactosidase-positive cells were positive for GABA in the hippocampus and neocortex, respectively (Fig. 2C–J), suggesting transgenic Vgat promoter-driven Cre-loxP recombination occurred globally in whole populations of GABAergic neurons. Conversely, 99.0% (1816 of 1835) and 88.5% (422 of 477) of β-galactosidase-positive cells were positive for GABA in the hippocampus and neocortex, respectively, suggesting transgenic Vgat promoter-driven Cre-loxP recombination may be restricted to global inhibitory neurons.

We next generated mice with heterozygous Nav1.1 deletion in global inhibitory neurons (Scn1a<sup>d/+</sup>,VGAT-Cre) by crossing Scn1a<sup>d/d</sup> and VGAT-Cre mice. PCR analysis of DNA from Scn1a<sup>d/d</sup>,VGAT-Cre whole brain verified VGAT-Cre-dependent recombination of the floxed Scn1a allele (Fig. 3A). Western blot analysis showed Nav1.1 expression in P21.5 Scn1a<sup>d/+</sup>, VGAT-Cre whole brain was reduced to 72.3 ± 3.4% level of that in Scn1a<sup>d+/+</sup> mice (Fig. 3B and C). Until the second postnatal...
week, Scn1a
fl/fl, VGAT-Cre mice were viable and physically indistinguishable from Scn1a
fl/+ mice, but at around P16 the mice developed spontaneous generalized convulsive seizures (Fig. 3D and E), which occasionally led to immediate death. In all three recorded lethal seizure cases, postictal electrocorticography (ECoG) activity progressively decreased, becoming undetectable within a minute after the end of the seizure (Fig. 3E). Maintenance of postictal ECoG activity until recovery from postictal immobility and ECoG suppression ensured generalized convulsions did not lead to death. Remarkably, all but one (64 of 65; 98.5%) Scn1a
fl/fl, VGAT-Cre mice died before P35 (Fig. 3F), and their under-P35 mortality rate was significantly higher than constitutive heterozygous Scn1a
d/+ mice (8 of 36; 22.2%).

We also generated mice with homozygous Nav1.1 deletion in global inhibitory neurons (Scn1a
fl/fl, VGAT-Cre) (see Materials and Methods) (Fig. 3G). Western blot analysis showed that Nav1.1 expression in P12.5 Scn1a
fl/fl, VGAT-Cre whole brain was reduced to 57.6 ± 9.9% level of that in Scn1a
fl/fl mice (Fig. 3H and I). Scn1a
fl/fl, VGAT-Cre mice were viable and showed no obvious abnormalities until around P10. However, after approximately P11, the mice exhibited behavioral hypoactivity, except for periodic myoclonus-like jerk movements, and all died before P15 (Fig. 3F). The average lifespan for Scn1a
fl/fl, VGAT-Cre mice (12.8 days; n = 4) was shorter than Scn1a
d/d mice (16.2 days; n = 19). Scn1a
fl/fl, VGAT-Cre mice did not develop severe ataxia and walked normally until a day before their death.

We used immunohistochemistry to examine the spatial pattern of Nav1.1 expression in P12.5 Scn1a
fl/fl, VGAT-Cre brain (Fig. 4), using an anti-Nav1.1 antibody whose specificity has been verified using Nav1.1-null mice as negative controls (15). P12.5 was the oldest age used because of the high mortality rate beyond P10 and the short life expectancy (within P15). In the hippocampus of Scn1a
fl/fl controls, Nav1.1-immunoreactive fibers and puncta were scattered throughout the CA fields (Fig. 4A). In contrast, in Scn1a
fl/fl, VGAT-Cre mice, Nav1.1-immunoreactive fibers and puncta were mostly undetectable (Fig. 4B). Similarly, in the neocortex, Nav1.1-immunoreactive fibers and puncta were scattered throughout neocortical layers II/III, IV, V and VI in Scn1a
fl/fl mice, but virtually undetected in Scn1a
fl/fl, VGAT-Cre mice (Fig. 4C and D).

In the cerebellum at P12.5, differences in Nav1.1 immunostaining patterns and intensities between Scn1a
fl/fl and Scn1a
fl/fl, VGAT-Cre mice were also observed (Fig. 4E and F), even though Scn1a
fl/fl, VGAT-Cre mice did not develop ataxia. Nav1.1-immunoreactive fibers in the inner molecular layer of the cerebellar lobes, presumably axons of cerebellar basket neurons, were reduced in Scn1a
fl/fl, VGAT-Cre mice (Fig. 4E and F), suggesting a reduced inhibitory input from the cerebellum to the hippocampal pyramidal neurons.
Selective Nav1.1 deletion in forebrain excitatory neurons does not cause epileptic seizures in mice

To determine whether excitatory neurons express Nav1.1, and if they do, what is their role in epileptic phenotypes, we employed the empty spiracles homolog 1 (Emx1)-Cre knock-in mouse expressing Cre recombinase under the control of the endogenous Emx1 promoter (26). Emx1 is a marker for cortical pyramidal cells and glia, and is not expressed in GABAergic cells (27,28). In the Emx1-Cre mouse, Cre recombinase is expressed in pallium-derived excitatory neurons in various brain regions, including the olfactory bulb, neocortex, piriform cortex, entorhinal cortex, hippocampus and amygdala (26).

PCR analysis of DNA from Scn1a<sup>fl/fl</sup>, Emx1<sup>-Cre</sup> whole brain verified Emx1-Cre-dependent recombination of the floxed Scn1a allele (Fig. 5A). Western blot analysis showed that Nav1.1 expression in Scn1a<sup>fl/fl</sup>, Emx1<sup>-Cre</sup> whole brain was reduced to 72.1 ± 1.7 and 63.4 ± 2.3% levels of those in Scn1a<sup>fl/fl</sup> mice at P21.5 and 8 weeks, respectively (Fig. 5B and C). Homozygous (Scn1a<sup>fl/fl</sup>, Emx1<sup>-Cre</sup>) mice were viable and with no noticeable abnormal phenotype, including no convulsive seizures (Fig. 5D).

Nav1.1 is expressed in subpopulations of excitatory neurons

The spatial and temporal patterns of Nav1.1 expression in Scn1a<sup>fl/fl</sup> and Scn1a<sup>fl/fl</sup>, Emx1<sup>-Cre</sup> brains were compared by...
immunohistochemistry. In the hippocampus at P21.5, Nav1.1-immunoreactive fibers and puncta were clearly observed in the stratum oriens and pyramidal layers of the CA fields, in both Scn1a^fl/fl and Scn1a^fl/fl, Emx1-Cre mice (Fig. 6A and B). In the neocortex at P21.5, Nav1.1-immunoreactive fibers and puncta appeared to be distributed similarly in Scn1a^fl/fl and Scn1a^fl/fl, Emx1-Cre mice; however, subtle differences were detected (Fig. 6C and D). In neocortical layer V of Scn1a^fl/fl controls, Nav1.1 immunoreactivity was detected in the proximity of neurites oriented toward the pial surface (upward), presumably AISs of PV interneurons, and also in the proximity of basal neurites oriented toward the ventricular surface (downward), of a
neuronal subpopulation with large Nav1.1-immunopositive somata (Fig. 6Cb). These Nav1.1-immunoreactive downward basal neurites were not observed in other neocortical layers. In contrast, in Scn1a<sup>fl/fl</sup>, Emx1-Cre mice, Nav1.1 immunoreactivity was occasionally detected in upward, but rarely in downward, neurites (Fig. 6Db). These observations suggest that cells with Nav1.1-positive downward basal neurites are excitatory pyramidal neurons. Therefore, we performed double-immunolabeling of neocortices in Gad67-GFP mice, expressing GFP in global inhibitory neurons (29), using anti-Nav1.1 and -GFP antibodies. We detected Nav1.1 immunoreactivity in proximal neurites of neocortical GFP-positive cells, as well as basal proximal neurites of a subpopulation of layer V GFP-negative cells with relatively large pyramidal-shaped somata (Supplementary Material, Fig. S2). These observations suggest that in the neocortex, Nav1.1 is expressed in axons and somata of a subpopulation of layer V pyramidal excitatory neurons, in addition to PV inhibitory neurons.

At postnatal week 8, differences in Nav1.1 immunoreactivity in the hippocampus and neocortex of Scn1a<sup>fl/fl</sup> and Scn1a<sup>fl/fl</sup>, VGAT-Cre mice became more apparent (Fig. 6E–J). In the hippocampus of Scn1a<sup>fl/fl</sup> mice, we observed a band of diffuse Nav1.1 immunoreactivity extending from the stratum lacunosum-moleculare within the CA fields to the dentate gyrus molecular layer (Fig. 6Ea), which would correspond to perforant path fibers arising from excitatory neurons of the entorhinal cortex, crossing the stratum lacunosum-moleculare in the hippocampal CA1 field and terminating in the lacunosum-moleculare in the hippocampal CA3 field or molecular layer of the dentate gyrus (30,31). As the middle one-third of the dentate gyrus molecular...
layer in Scn1a<sup>fl/fl</sup> controls was clearly Nav1.1-immunoreactive, excitatory neurons in the medial (rather than the lateral) entorhinal cortex appear to express Nav1.1 in their perforant afferents (31,32). In Scn1a<sup>fl/fl</sup>, Emx1-Cre mice, Nav1.1 immunoreactivity in the perforant path was reduced compared with Scn1a<sup>+/+</sup> controls (Fig. 6Fa), whereas Nav1.1 signals in axon arborizations of PV basket cells were largely maintained (Fig. 6Eb and Fb). These observations, together with the occurrence of Cre-loxP recombination in excitatory neurons in the entorhinal cortex in the Emx1-Cre driver line (26), suggest Nav1.1 is expressed in the perforant path of medial entorhinal cortex excitatory neurons.

Previous studies have shown that hippocampal pyramidal cells and dentate gyrus granule cells express Nav1.1 in their somata and dendrites (14,18,33,34). In contrast, other studies failed to detect Nav1.1 in somata and proximal neurites of most, if not all, hippocampal and dentate gyrus excitatory cells (15,35–37). In agreement with the latter studies, our immunohistochemical analysis also failed to detect any distinctive Nav1.1 immunoreactivity in somata and proximal neurites of hippocampal and dentate gyrus excitatory neurons in Scn1a<sup>fl/fl</sup> mice (Fig. 6A and E), even when compared with Scn1a<sup>+/+</sup>, Emx1-Cre mice (Fig. 6B and F).

In the neocortex at postnatal week 8, diffuse Nav1.1 immunoreactivity in layers II/III and V was lower in Scn1a<sup>fl/fl</sup>, Emx1-Cre mice (Fig. 6H), compared with Scn1a<sup>+/+</sup> controls (Fig. 6G). Nav1.1-immunoreactive AIS-like fibers were not found in any neocortical layers from either genotype. Axons of excitatory neurons in neocortical layers II/III and V arborize in layers II/III and V (38–40); therefore, our results may suggest these axonal arborizations express Nav1.1.

We also performed immunohistochemical analysis on coronal brain sections of 8-week-old Scn1a<sup>fl/fl</sup> and Scn1a<sup>+/+</sup>, Emx1-Cre mice (Fig. 6I and J). Diffuse Nav1.1 immunoreactivity in the corpus callosum was observed in Scn1a<sup>fl/fl</sup> controls and was reduced in Scn1a<sup>fl/fl</sup>, Emx1-Cre mice, suggesting that Nav1.1 is expressed in callosal axons of neocortical excitatory neurons. In Scn1a<sup>fl/fl</sup> mice, we also detected columnar structures of diffuse Nav1.1 immunosignal in layer IV and at the boundary between layers V and VI, in barrel fields of somatosensory cortex (Fig. 6Ib), where axons of thalamo-cortical excitatory projection neurons arborize (41), suggesting Nav1.1 localizes in these axonal arborizations. In Scn1a<sup>+/+</sup>, Emx1-Cre mice, diffuse Nav1.1 immunoreactivities in neocortical layers II/III and V were reduced, yet the distinctive Nav1.1-immunoreactive columnar structures still detected (Fig. 6Ib), consistent with the previous observation that no Cre-loxP recombination was detected in thalamo-cortical neurons in the Emx1-Cre driver line (26).

Additional Nav1.1 deletion in excitatory neurons of mice with Nav1.1 haploinsufficiency in global inhibitory neurons ameliorates seizure-related sudden death

It was surprising that Scn1a<sup>fl/+</sup>, VGAT-Cre mice show a greater premature mortality rate than constitutional heterozygous Scn1a knock-out mice (for a comparison, see Figs 1F and 3F), raising the possibility that remaining Nav1.1 in excitatory neurons of Scn1a<sup>fl/+</sup>, VGAT-Cre mice may have aggravating effects on seizure-related sudden death. In order to investigate this possibility, we generated triple-heterozygous (Scn1a<sup>fl+</sup>, Emx1-Cre, VGAT-Cre) mice. Like Scn1a<sup>fl+/+</sup>, VGAT-Cre mice, Scn1a<sup>fl+/+</sup>, Emx1-Cre, VGAT-Cre mice were viable until the end of the second postnatal week, developing convulsive seizures in the third postnatal week. Importantly, however, the
under-P35 mortality rate in Scn1a\textsuperscript{fl/+}, Emx1-Cre, VGAT-Cre mice (15 of 38; 39.5%) was much lower than Scn1a\textsuperscript{fl/+}, VGAT-Cre mice (40 of 41; 97.7%), and similar to the rate in Scn1a\textsuperscript{d/+} mice (8 of 36; 22.2%) (Fig. 7). This suggests that Nav1.1 haploinsufficiency in Emx1-lineage forebrain excitatory neurons reduces the risk of seizure-related sudden death.

Selective Nav1.1 deletion in PV cells causes spontaneous epileptic seizures and ataxia in mice

To selectively delete Nav1.1 in PV cells, we employed a PV-Cre driver line, Pvalb-Cre BAC transgenic (PV-Cre-TG) mice, in which Cre-loxP recombination occurs in the majority of PV cells (42). PCR analysis of DNA from Scn1a\textsuperscript{fl/fl}, PV-Cre-TG
whole brain verified PV-Cre-TG-dependent recombination of the floxed Scn1a allele (Fig. 8A). Western blot analysis showed that Nav1.1 expression in P21.5 Scn1a<sup>fl/fl</sup>,PV-Cre-TG whole brain was reduced to 51.4 ± 2.1% level of that in Scn1a<sup>fl/fl</sup> littermates (Fig. 8B and C). Homozygotes (Scn1a<sup>fl/fl</sup>, PV-Cre-TG) were viable with no obvious abnormal phenotypes in the first postnatal week. However, they developed ataxia and spontaneous generalized convulsive seizures around P10 and P14, respectively (Fig. 8D and E), and all died before P30 (Fig. 8F). Heterozygotes (Scn1a<sup>fl/+</sup>,PV-Cre-TG) were viable until the second postnatal week, but some developed recurrent seizures and suffered sporadic sudden death after P16 (Fig. 8F). Thus, the phenotypes of homozygote and heterozygote mice are similar to, or even milder (for example, showing delayed seizure onset) than, those of previously reported constitutional Nav1.1-homozygous and heterozygous knock-out mice, respectively (14,15).

Immunohistochemical analysis on the hippocampus and neocortex of Scn1a<sup>fl/fl</sup>,PV-Cre-TG and Scn1a<sup>fl/fl</sup> mice at P12.5 (before the development of epileptic seizures in Scn1a<sup>fl/fl</sup>,PV-Cre-TG mice) detected weak Nav1.1 immunoreactivities on fibers and puncta, scattered within the stratum oriens, pyramidale and radiatum of hippocampal CA fields, as well as in neocortical layers II/III, IV, V and VI. The patterns and intensities of the antibody signals showed no obvious genotype differences (Fig. 9A–D), and provide a likely explanation for the lack of seizure development in Scn1a<sup>fl/fl</sup>,PV-Cre-TG, compared with Scn1a<sup>dd</sup> mice, at this developmental stage. It also suggests that transgenic PV promoter-driven Cre-loxP recombination in the hippocampus and neocortex scarcely occurs at P12.5, consistent with previous reports that PV expression in hippocampal and neocortical cells begins in the second and third postnatal weeks (43–45).

At P16.5, when seizures begin in Scn1a<sup>fl/fl</sup>,PV-Cre-TG mice, intensities of hippocampal Nav1.1 immunosignals appeared moderately reduced in Scn1a<sup>fl/fl</sup>,PV-Cre-TG mice, compared with age-matched Scn1a<sup>dd</sup> mice (Fig. 9E and F). In contrast, neocortical Nav1.1 immunostaining intensities and patterns remained similar between genotypes (Fig. 9G and H).

At P21.5, when Scn1a<sup>fl/fl</sup>,PV-Cre-TG mice suffer recurrent seizures and sporadic sudden death, genotype differences in Nav1.1 immunostaining patterns and intensities in the
hippocampus and neocortex became more apparent (Fig. 9I–L). In the hippocampus, Nav1.1-immunoreactive fibers and puncta in CA fields, presumably axons of PV basket cells, were strongly detected in Scn1a<sup>fl/fl</sup>, but almost absent in Scn1a<sup>fl/fl</sup>, PV-Cre-TG mice (Fig. 9I and J). In the neocortex, Nav1.1 immunoreactivity was often localized in neurites oriented toward the pial surface in Scn1a<sup>fl/fl</sup> mice, but this localization was not distinctly detected in Scn1a<sup>fl/fl</sup>, PV-Cre-TG mice (Fig. 9K–N), providing further evidence that neocortical PV cells express Nav1.1 (15). In contrast, fibrous Nav1.1 immunoreactivity was occasionally detected in downward projecting basal neurites of layer V cells in both Scn1a<sup>fl/fl</sup> and Scn1a<sup>fl/fl</sup>, PV-Cre-TG mice (Fig. 9M and N). Taken together with our observations that downward projecting basal neurites with Nav1.1 immunoreactivity were rarely observed in Scn1a<sup>fl/fl</sup>, Emx1-Cre mice (see Fig. 6Db), Nav1.1 appears to be expressed in a subset of neocortical layer V PV-negative, excitatory pyramidal neurons, consistent with recent studies (18,19). It is notable that the diffuse background-like signal, presumably a signal in distal axons of excitatory neurons (see also Fig. 6I and J), remained high, or became even higher, in both genotypes compared with those at P16.5. In addition, Nav1.1 immunoreactivities at neocortical layer IV, and between layers V and VI, reflecting signals in arborized axons of thalamo-cortical projection neurons (41), and reported as PV-positive (42), were moderately reduced in Scn1a<sup>fl/fl</sup>, PV-Cre-TG mice, compared with Scn1a<sup>fl/fl</sup> mice (Fig. 9Ka and La).

Immunofluorescent examination of Nav1.1 colocalization with PV and ankyrin-G, a marker for AISs, in P21.5 Scn1a<sup>fl/fl</sup> controls, confirmed marked Nav1.1 immunopositivity in AISs of neocortical PV cells (Fig. 9O), reported previously at P14–16 (15). In contrast, a significant reduction in Nav1.1 immunostaining intensities was observed in AISs of most neocortical PV cells in P21.5 Scn1a<sup>fl/fl</sup>, PV-Cre-TG mice (Fig. 9P).

Figure 7. Additional Emx1-Cre-mediated Nav1.1 deletion in Scn1a<sup>+/+</sup>, VGAT-Cre mice ameliorates seizure-related sudden death. Survival curves of P3 Scn1a<sup>+/+</sup>, Scn1a<sup>+/+</sup>, Emx1-Cre, Scn1a<sup>+/+</sup>, Emx1-Cre, VGAT-Cre and Scn1a<sup>–/–</sup>, VGAT-Cre littermates. Survival curve of Scn1a<sup>–/–</sup> (reprinted from Fig. 1F) is included for comparison. Note that a significant decrease in premature lethality in Scn1a<sup>–/–</sup>, Emx1-Cre, VGAT-Cre mice was observed in comparison with Scn1a<sup>+/+</sup>, VGAT-Cre mice. +, wild-type allele; fl, floxed allele.

Figure 8. Selective Nav1.1 deletion in mouse PV cells by PV-Cre causes spontaneous epileptic seizures and premature death. (A) Verification of PV-Cre-TG-dependent floxed Scn1a gene deletion in the brain by PCR analysis. (B and C) Verification of PV-Cre-TG-dependent Nav1.1 deletion by semi-quantitative western blot analysis on brain membrane proteins prepared from P21.5 Scn1a<sup>+/+</sup>, PV-Cre-TG and Scn1a<sup>+/+</sup> littermates, using anti-Nav1.1 antibody. Error bars represent SEM; two independent assays were performed. (D and E) Representative interictal (D) and ictal (E) ECoG recordings in P14–16 Scn1a<sup>+/+</sup>, PV-Cre-TG mice and Scn1a<sup>+/+</sup> control mice (n = 3, each group). (F) Survival curves of P3 Scn1a<sup>+/+</sup>, Scn1a<sup>+/+</sup>, PV-Cre-TG and Scn1a<sup>+/+</sup>, PV-Cre-TG littermates. Note that all Scn1a<sup>+/+</sup>, PV-Cre-TG mice died before P25. +, wild-type allele; fl, floxed allele. ***P < 0.001.
Figure 9. Nav1.1 immunosignals in PV inhibitory neurons are reduced at later developmental stages in Scn1a\(^{fl/fl}\), PV-TG-Cre mice. (A–N) Representative parasagittal sections of P12.5 Scn1a\(^{fl/fl}\) (A) and Scn1a\(^{fl/fl}\), PV-Cre-TG (B) hippocampi, P12.5 Scn1a\(^{fl/fl}\) (C) and Scn1a\(^{fl/fl}\), PV-Cre-TG (D) neocortices, P16.5 Scn1a\(^{fl/fl}\) (E) and Scn1a\(^{fl/fl}\), PV-Cre-TG (F) hippocampi, P16.5 Scn1a\(^{fl/fl}\) (G) and Scn1a\(^{fl/fl}\), PV-Cre-TG (H) neocortices, P21.5 Scn1a\(^{fl/fl}\) (I) and Scn1a\(^{fl/fl}\), PV-Cre-TG (J) hippocampi and P21.5 Scn1a\(^{fl/fl}\) (K and M) and Scn1a\(^{fl/fl}\), PV-Cre-TG (L and N) neocortices, stained with anti-Nav1.1 antibody. P21.5 was the oldest age used for analysis as Scn1a\(^{fl/fl}\), PV-TG-Cre mice show a high mortality rate beyond P15 and a life expectancy within P30. Higher magnification images outlined in (a) are shown in (b). Arrowheads indicate putative Nav1.1-immunoreactive AISs of neocortical PV cells. Double arrowheads indicate Nav1.1-immunoreactive proximal neurites oriented toward the ventricular surface. Scale bars: (a) 400 \(\mu\)m; (b) 40 \(\mu\)m. DG, dentate gyrus; o, stratum oriens; p, stratum pyramidale; l, stratum lucidum. (O and P) Immunofluorescence histochemistry of neocortices of P21.5 Scn1a\(^{fl/fl}\) (O) and Scn1a\(^{fl/fl}\), PV-TG-Cre (P) mice stained with anti-Nav1.1 (a; red), PV (b; green) and ankyrin G (c; cyan) antibodies and counterstained with DAPI (d; blue). Merged images are shown (e). Arrowheads indicate AISs of neocortical PV cells with strong Nav1.1 immunofluorescence. An arrow indicates an AIS of a PV cell with reduced Nav1.1 immunofluorescence. Scale bars: 100 \(\mu\)m. (Q and R) Representative parasagittal sections of P12.5 Scn1a\(^{fl/fl}\) (Q) and Scn1a\(^{fl/fl}\), PV-TG-Cre (R) cerebellum stained with anti-Nav1.1 antibody. Higher magnification images outlined in (a) are shown in (b) and (d). Higher magnification images outlined in (b) are shown in (c). Scale bars: (a) 400 \(\mu\)m; (b and c) 40 \(\mu\)m; (d) 80 \(\mu\)m. Arrowheads and arrows indicate Nav1.1-immunoreactive AISs of Purkinje cells and axons of basket cells, respectively. M, molecular cell layer; P, Purkinje cell layer; G, granule cell layer; CbN, cerebellar nuclei. Nuclear immunosignals are non-specific (15). Images are oriented from the pial surface (top) to the callosal (bottom), and from rostral (left) to caudal (right). fl, floxed allele.
In the cerebellum at P12.5, when Scn1a<sup>fl/fl</sup>, PV-Cre-TG mice suffer severe ataxia, differences in Nav1.1 immunostaining patterns and intensities were observed between Scn1a<sup>fl/fl</sup> and Scn1a<sup>fl/fl</sup>, PV-Cre-TG mice (Fig. 9Q and R). Nav1.1-immunoreactive fibers and puncta distributed in the inner molecular layer of cerebellar lobes, presumably corresponding to PV-expressing cerebellar basket cells (15,46), were clearly observed in Scn1a<sup>fl/fl</sup>, but virtually undetectable in Scn1a<sup>fl/fl</sup>, PV-Cre-TG mice (Fig. 9Qa–c and Ra–c). Similarly, immunoreactivities in AISs of Purkinje cells were observed in Scn1a<sup>fl/fl</sup> mice, as previously reported (15), but not in Scn1a<sup>fl/fl</sup>, PV-Cre-TG mice (Fig. 9Qc and Rc). Intensities of Nav1.1 immunoreactivities on puncta scattered within cerebellar nuclei and white matter, comprising projecting axons from cerebellar Purkinje cells (47), were reduced in Scn1a<sup>fl/fl</sup>, PV-Cre-TG mice, compared with Scn1a<sup>fl/fl</sup> mice (Fig. 9Qd and Rd).

We also crossed floxed Scn1a mice with Pvalb-Cre knock-in (PV-Cre-KI) mice (48), in which the PV-Cre knock-in allele harbors an internal ribosome entry site (IRES) and Cre recombinase inserts into the 3′ UTR of the mouse Pvalb gene. Endogenous Pvalb promoter-driven Cre-loxP recombination occurs only
within PV inhibitory neurons, with high PV expression suggesting low recombination efficiency (49–51).

PCR analysis of DNA from Scn1a^fl/fl, PV-Cre-KI whole brain verified PV-Cre-KI-dependent floxed Scn1a gene deletion in Scn1a^fl/fl, PV-Cre-KI brain by PCR analysis. (B and C) Verification of PV-Cre-KI-dependent Nav1.1 deletion by semi-quantitative western blot analysis of brain membrane proteins prepared from P21.5 and 8-week-old Scn1a^fl/fl, PV-Cre-KI and Scn1a^fl/fl littermates, using anti-Nav1.1 antibody (B). Mean Nav1.1 expression levels in Scn1a^fl/fl, PV-Cre-KI mice are represented as percentages relative to Scn1a^fl/fl mice (100%) (C). Error bars represent SEM; two independent assays were performed. (D–F) Spontaneous epileptic seizures in Scn1a^fl/fl, PV-Cre-KI mice. Representative normal interictal (D), ictal (E) and abnormal interictal (F) ECoGs recorded in Scn1a^fl/fl, PV-Cre-KI mice are shown. ECoG recordings were performed with P25–30 Scn1a^fl/fl, PV-Cre-KI and Scn1a^fl/fl control mice. (G) Survival curves of P3 Scn1a^fl/fl, Scn1a^+/+, Scn1a^+/+, PV-Cre-KI and Scn1a^fl/fl, PV-Cre-KI littermates. A proportion of Scn1a^fl/fl, PV-Cre-KI mice suffered sporadic death after P25. +, wild-type allele; fl, floxed allele.

Figure 10. Minimal selective Nav1.1 deletion in PV cells by low-efficiency PV-Cre recombination is sufficient to cause spontaneous epileptic seizures and occasional premature death in mice. (A) Verification of PV-Cre-KI-dependent floxed Scn1a gene deletion in Scn1a^fl/fl, PV-Cre-KI brain by PCR analysis. (B and C) Verification of PV-Cre-KI-dependent Nav1.1 deletion by semi-quantitative western blot analysis of brain membrane proteins prepared from P21.5 and 8-week-old Scn1a^fl/fl, PV-Cre-KI and Scn1a^fl/fl littermates, using anti-Nav1.1 antibody (B). Mean Nav1.1 expression levels in Scn1a^fl/fl, PV-Cre-KI mice are represented as percentages relative to Scn1a^fl/fl mice (100%) (C). Error bars represent SEM; two independent assays were performed. (D–F) Spontaneous epileptic seizures in Scn1a^fl/fl, PV-Cre-KI mice. Representative normal interictal (D), ictal (E) and abnormal interictal (F) ECoGs recorded in Scn1a^fl/fl, PV-Cre-KI mice are shown. ECoG recordings were performed with P25–30 Scn1a^fl/fl, PV-Cre-KI and Scn1a^fl/fl control mice. (G) Survival curves of P3 Scn1a^fl/fl, Scn1a^+/+, Scn1a^+/+, PV-Cre-KI and Scn1a^fl/fl, PV-Cre-KI littermates. A proportion of Scn1a^fl/fl, PV-Cre-KI mice suffered sporadic death after P25. +, wild-type allele; fl, floxed allele.

within PV inhibitory neurons, with high PV expression suggesting low recombination efficiency (49–51).

PCR analysis of DNA from Scn1a^fl/fl, PV-Cre-KI whole brain verified PV-Cre-KI-dependent recombination of the floxed Scn1a allele (Fig. 10A). Western blot analysis showed that Nav1.1 expression levels in Scn1a^fl/fl, PV-Cre-KI mouse whole brain at P21.5 and 8 weeks were 98.9 ± 6.4 and 87.6 ± 4.3% of those in age-matched Scn1a^fl/fl control littersmates, respectively, although the slight reduction at 8 weeks was not statistically significant (Fig. 10B and C). Scn1a^fl/fl, PV-Cre-KI mice were viable with no obvious abnormal phenotypes until the beginning of the third postnatal week. The mice developed spontaneous recurrent convulsive seizures with abnormal ECoG patterns around P20 (Fig. 10D and E). In addition, the mice exhibited abnormal interictal poly-spike-wave discharges, which were most pronounced during resting or sleeping (Fig. 10F). At around P20, Scn1a^fl/fl, PV-Cre-KI mice developed ataxia, which was clearly observed in inclined (an ~45° angle) cages. Scn1a^fl/fl mice walked up and down the slope with a steady gait; however, in contrast, although Scn1a^fl/fl, PV-Cre-KI mice were able to climb up the slope, they always slipped down the slope with an unstable gait. Some Scn1a^fl/fl, PV-Cre-KI mice suffered sporadic sudden death after P25 (Fig. 10G). These results suggest that a minimal amount of Nav1.1 deletion, specifically in PV cells, is enough to cause spontaneous epileptic seizures, ataxia and occasional sudden death.

The spatial and temporal patterns of Nav1.1 expression were examined by immunohistochemistry in Scn1a^fl/fl, PV-Cre-KI brain parasagittal sections. At P16.5, before Scn1a^fl/fl, PV-Cre-KI mice developed epileptic seizures, Nav1.1 immunostaining intensities and patterns in the hippocampus and neocortex of Scn1a^fl/fl, PV-Cre-KI mice were similar to those in age-matched Scn1a^+/+ controls (Fig. 11A–D). This is in agreement with late onset of endogenous PD promoter-driven Cre-loxP recombination in the hippocampus and neocortex (52), and provides a plausible explanation for the lack of seizures in Scn1a^fl/fl, PV-Cre-KI mice at this postnatal stage, despite Scn1a^+/+ mice of the same age suffering recurrent seizures (see Fig. 1D and E). In Scn1a^fl/fl mice,
Figure 11. Nav1.1 immunosignals in PV inhibitory neurons are moderately reduced at later developmental stages in Scn1a<sup>fl/fl</sup>, PV-KI-Cre mice. (A–L) Representative parasagittal sections of P16.5 Scn1a<sup>fl/fl</sup> (A) and Scn1a<sup>fl/fl</sup>, PV-Cre-KI (B) hippocampi, P16.5 Scn1a<sup>fl/fl</sup> (C) and Scn1a<sup>fl/fl</sup>, PV-Cre-KI (D) neocortices, P21.5 Scn1a<sup>fl/fl</sup> (E) and Scn1a<sup>fl/fl</sup>, PV-Cre-KI (F) hippocampi, P21.5 Scn1a<sup>fl/fl</sup> (G) and Scn1a<sup>fl/fl</sup>, PV-Cre-KI (H) neocortices, 8-week-old Scn1a<sup>fl/fl</sup> (I) and Scn1a<sup>fl/fl</sup>, PV-Cre-KI (J) hippocampi and 8-week-old Scn1a<sup>fl/fl</sup> (K) and Scn1a<sup>fl/fl</sup>, PV-Cre-KI (L) neocortices, stained with anti-Nav1.1 antibody. Higher magnification images outlined in (a) are shown in (b). Scale bars: (a) 400 μm; (b) 40 μm. Arrowheads and double arrowheads indicate Nav1.1-immunoreactive AISs of neocortical PV cells and hippocampal perforant path afferents, respectively. DG, dentate gyrus; o, stratum oriens; p, stratum pyramidale; l, stratum lucidum; r, stratum radiatum; lm, stratum lacunosum-moleculare; m, dentate gyrus molecular layer; g, dentate gyrus granule cell layer. (M and N) Representative parasagittal sections of P21.5 Scn1a<sup>fl/fl</sup> (M) and Scn1a<sup>fl/fl</sup>, PV-Cre-KI (N) cerebellum, stained with anti-Nav1.1 antibody. Higher magnification images outlined in (a) are shown in (b) and (d). Higher magnification images outlined in (b) are shown in (c). Arrows indicate putative Nav1.1-immunoreactive axons of basket cells. Scale bars: (a) 400 μm; (b and c) 40 μm; (d) 80 μm. M, molecular cell layer; P, Purkinje cell layer; G, granule cell layer; ChN, cerebellar nuclei. Nuclear Nav1.1 immunostaining is non-specific (15). Images are oriented from the pial surface (top) to the callosal (bottom), and from rostral (left) to caudal (right). fl, floxed allele.
Nav1.1 immunoreactivities in the neocortex were found in AISs of neocortical PV cells that often oriented toward the pial surface, as previously described (15,36) (see also Fig. 9O).

At P21.5, when Scn1a<sup>fl/fl</sup> PV-Cre-KI mice began to suffer recurrent seizures, Nav1.1-immunoreactive fibers in hippocampal CA fields, presumably axons of PV-positive fast-spiking basket cells (15), were observed in Scn1a<sup>fl/fl</sup> mice (Fig. 11E), but reduced in Scn1a<sup>fl/fl</sup>, PV-Cre-KI mice (Fig. 11F). This is consistent with Nav1.1 localization in axons of hippocampal PV inhibitory neurons within the stratum oriens and pyramidale (53–55) in wild-type mice (15,37). In contrast, staining intensities and patterns in Nav1.1-immunoreactive fibers and puncta scattered within the neocortex appeared similar between genotypes at this stage (Fig. 11G and H), suggesting that modest reductions in Nav1.1 expression levels in hippocampal PV interneurons are sufficient to cause epileptic seizures in mouse.

At postnatal week 8, differences in Nav1.1 immunostaining patterns in the hippocampus of Scn1a<sup>fl/fl</sup>, PV-Cre-KI and Scn1a<sup>fl/fl</sup> mice became more evident (Fig. 11I and J). Nav1.1-immunoreactive fibers and puncta scattered in hippocampal CA fields were observed in Scn1a<sup>fl/fl</sup> mice, but virtually undetected in Scn1a<sup>fl/fl</sup>, PV-Cre-KI mice. Moreover, the band of diffuse Nav1.1 immunoreactivity extending from the stratum lacunosum-moleculare of hippocampal CA fields to the molecular layer of the dentate gyrus was detected similarly in both genotypes (Fig. 11Ia and Ja). These diffuse Nav1.1 immunoreactivities are assumed to be the perforant path (30,31) (see also Fig. 6Ea and Fa). In the neocortex at postnatal week 8, overall Nav1.1 immunostaining intensities appeared subtly decreased in Scn1a<sup>fl/fl</sup>, PV-Cre-KI mice, compared with Scn1a<sup>fl/fl</sup> mice (Fig. 11K and L).

In the cerebellum at P21.5, a stage when Scn1a<sup>fl/fl</sup>, PV-Cre-KI mice display ataxia, differences in Nav1.1 immunostaining intensities were observed between Scn1a<sup>fl/fl</sup> and Scn1a<sup>fl/fl</sup>, PV-Cre-KI mice (Fig. 11M and N). Nav1.1 immunoreactivities in fibers and puncta distributed in the inner molecular layer of cerebellar lobes, putatively corresponding to axons of PV-expressing cerebellar basket cells (15,46), appeared weaker in Scn1a<sup>fl/fl</sup>, PV-Cre-KI mice than in Scn1a<sup>fl/fl</sup> mice (Fig. 11Ma and Na). Also, Nav1.1-immunoreactive puncta scattered in cerebellar nuclei and white matter, comprising PV-expressing cerebellar Purkinje cells axons (47), appeared reduced in Scn1a<sup>fl/fl</sup>, PV-Cre-KI mice, compared with Scn1a<sup>fl/fl</sup> mice (Fig. 11Md and Nd).

All of the phenotypic and immunohistochemical data on a series of conditional Scn1a mutant mice obtained in the present study were summarized in Tables 1 and 2.

**DISCUSSION**

Our study on a series of conditional Scn1a knock-out mice provides important insights into the pathology of Dravet syndrome. Mice with selective Nav1.1 deletion in global inhibitory neurons (using VGAT-Cre recombination) suffered epileptic seizures with a significantly higher risk of lethality than constitutive Scn1a knock-out mice. Nav1.1 deletion in mouse excitatory neurons (using Emx1-Cre recombination) did not cause epileptic seizures or any other observable behavioral abnormalities, but ameliorated the significant lethality of Nav1.1 deletion in global inhibitory neurons (using combined Emx1-Cre and VGAT-Cre-dependent recombination) (Tables 1 and 2). These findings show for the first time the protective or ameliorating effect of Nav1.1 haploinsufficiency in excitatory neurons on the risk of SUDEP in Dravet syndrome.

Cheah et al. (19) recently showed inhibitory neuron-specific Nav1.1 deletion in mice, using the Dlx1/2-I12b-Cre driver (Scn1a<sup>B+/Dlx-Cre</sup>), which caused spontaneous epileptic seizures and premature lethality, although the phenotype of Scn1a<sup>B+</sup>, Dlx-Cre mice was milder than that of Scn1a<sup>B+</sup>, VGAT-Cre mice, and more comparable with constitutive heterozygous Scn1a knock-out mice. Dlx1/2-I12b-Cre expresses Cre recombinase in the majority of GABAergic inhibitory neurons, including PV-, somatostatin-, neuropeptide Y- and calretinin-positive cells (20). Given that Dlx1/2-I12b-Cre-mediated Nav1.1 deletion occurs in ~50% of forebrain inhibitory neurons (19), phenotypic differences between Scn1a<sup>B+</sup>, Dlx-Cre and Scn1a<sup>B+</sup>, VGAT-Cre mice are likely due to differences in Nav1.1 deletion efficiency in inhibitory neurons. Moreover, differences in genetic backgrounds (Scn1a<sup>B+</sup>, Dlx-Cre and Scn1a<sup>B+</sup>, VGAT-Cre mice on C57BL/6/CD1 and C57BL/6/129 mixed backgrounds, respectively) may have affected severities of premature lethality.
(14,15). The study on Scn1a<sup>B+</sup>, Dlx-Cre mice (19) also found Nav1.1 expression in certain excitatory neurons, including layer V pyramidal cells; however, they did not discuss a functional role of Nav1.1 in excitatory neurons, with regard to epileptic phenotypes.

Dutton et al. (18) reported another conditional Nav1.1 inactivation in mice, using the Ppp1r2-Cre driver that preferentially induces Cre-loxP recombination in a subset of forebrain GABAergic inhibitory neurons consisting of ~75, 30 and 15% of total PV-, reelin- and neuropeptide Y-positive cells (21). Scn1a<sup>B+</sup>, Ppp1r2-Cre mice show increased flurothyl- and hyperthermia-induced seizure susceptibilities, and develop infrequent seizures. However, the mice do have a normal lifespan and less spontaneous seizures compared with constitutive Scn1a knock-out mice (18). This milder phenotype could be due to restricted or incomplete Nav1.1 deletion by Ppp1r2-Cre, as discussed by the authors (18). Dutton et al. (18) also generated Scn1a<sup>B+</sup>, Emx1-Cre mice, reporting that the mice had normal susceptibility levels of flurothyl- and hyperthermia-induced seizures, not consistent with our observation of an ameliorating effect of Emx1-Cre-dependent Nav1.1 deletion in Scn1a<sup>B</sup>, VGAT-Cre mice. It is possible that the protective effect of Nav1.1-haploinsufficient forebrain excitatory neurons is only observed when seizures are generated spontaneously or in distinct pathologies, and may partially explain the conflicting results. Alternatively, seizure generation and seizure-related sudden death may involve distinct neuronal circuits or networks. Further studies are needed to investigate the nature of these discrepancies.

Our study also showed that minimal PV-positive cell-specific Nav1.1 deletion, using PV-Cre-KI, is sufficient to cause spontaneous epileptic seizures and ataxia in mice (Tables 1 and 2), further supporting our previous proposal that functional impairment of PV interneurons is the basis for epileptic seizures in Dravet syndrome (15). PV has been widely used as a specific biochemical marker for a subclass of forebrain GABAergic inhibitory neurons that can also be grouped electro-physiologically as fast-spiking neurons, or morphologically as basket cells and chandelier cells. In Pvalb-IRESCre lines, for example, the PV-Cre-KI mice used in our study, endogenous Pvalb promoter-driven Cre-loxP recombination occurs only in subsets of PV inhibitory neurons expressing high PV levels and with large somata (49), suggesting incomplete Nav1.1 deletion in PV inhibitory neurons of Scn1a<sup>B</sup>, PV-Cre-KI mice. In the PV-Cre-TG driver line, transgenic Pvalb promoter-driven Cre-loxP recombination occurs in the majority of PV cells and, to a much lesser extent, in PV-negative cells, including a subpopulation of somatostatin-positive inhibitory neurons (42). Higher efficiency or more widespread occurrence of Cre-loxP recombination in PV-Cre-TG, than PV-Cre-KI, driver line likely accounts for the greater amount of PV-Cre-dependent Nav1.1 reduction in Scn1a<sup>B</sup>, PV-Cre-TG mice than in Scn1a<sup>B</sup>, PV-Cre-KI mice (Tables 1 and 2). Differences in Cre-loxP recombination efficiency in PV inhibitory neurons appear to contribute to differences in phenotypic severity between Scn1a<sup>B</sup>, PV-Cre-TG and Scn1a<sup>B</sup>, PV-Cre-KI mice. The phenotypic severity of Scn1a<sup>B</sup>, PV-Cre-TG mice is comparable with constitutive Scn1a heterozygous knock-out mice, but far milder than Scn1a<sup>B</sup>, VGAT-Cre mice (Tables 1 and 2). Several explanations can account for these differences. First, the onset of PV expression occurs at a later developmental stage. In hippocampal and neocortical PV interneurons, PV expression is detectable in the second postnatal week and progressively up-regulated thereafter (43–45; Allen Developing Mouse Brain, http://developingmouse.brain-map.org/). Coincident with the onset and up-regulation of PV expression, Nav1.1 expression levels in the hippocampus and neocortex of Scn1a<sup>B</sup>, PV-Cre-TG mice are progressively reduced after the second postnatal week. Compared with Scn1a<sup>B</sup>, PV-Cre-TG mice, Scn1a<sup>B</sup>, VGAT-Cre mice had an earlier onset of a reduction in Nav1.1 expression levels in both the hippocampus and neocortex, consistent with RNA in situ hybridization data showing detectable VGAT expression in the forebrain in the first postnatal week, before the onset of PV expression (Allen Developing Mouse Brain, http://developingmouse.brain-map.org/). Second, the milder phenotype observed in Scn1a<sup>B</sup>, PV-Cre-TG mice may be facilitated by the ameliorating effect of Nav1.1 deletion in PV-positive excitatory neurons. In addition to PV interneurons, PV expression is found in several excitatory neuronal subtypes, including a small subpopulation of neocortical layer V excitatory neurons and thalamo-cortical excitatory projection neurons (42,49,56). Our study shows Nav1.1

![Table 1. Summary of phenotypes observed in conditional Scn1a mutant mice generated in this study](https://example.com/table1.png)

| Genotype driver (Cre) | Scn1a alleles | Seizure (onset age) | Ataxia (onset age) | Average lifespan | Under-P35 mortality | Genetic background |
|-----------------------|---------------|---------------------|-------------------|-----------------|---------------------|-------------------|
| (Ella-Cre)<sup>a</sup> | fl/fl, Scn1a<sup>B</sup>, VGAT-Cre | ND 15.4% (∼P10) | ND 22.2% (∼P16) | ND 98.6% (∼P16) | ND 97.7% (∼P18) | ND 95.0% (∼P20) |
| d/d                  | GTC (~P12)   | Severe (~P10)       | 16.2 days (~P10)  | 100% (~P10)     | 100% (~P10)        | 0.97:0.03          |
| VGAT-Cre             | fl/fl        | Myoclonus (~P11)    | 12.8 days (~P4)   | 100% (~P4)      | 96.0:04            | ND 91.0% (∼P14)   |
|                       | fl/+         | GTC (~P16)          | ND               | 98.6% (~P65)    | 94.0:06            | ND 91.0% (~P18)   |
| Emx1-Cre             | fl/fl        | ND                  | ND               | ND 15.4% (~P13) | 91.0:09            | ND 91.0% (~P20)   |
| Emx1-Cre, VGAT-Cre   | fl/fl        | ND                  | ND               | ND 39.5% (~P38) | 95.0:05            | ND 91.0% (~P33)   |
| PV-Cre-TG            | fl/fl        | ND                  | ND               | ND 3.8% (~P53)  | ND 91.0:09         | ND 91.0% (~P53)   |
| PV-Cre-KI            | fl/fl        | Mild (~P20)         | ND               | 15.6% (~P32)    | 91.0:09            | ND 91.0% (~P32)   |
|                       | fl/+         | GTC (~P20)          | ND               | 15.6% (~P32)    | 91.0:09            | ND 91.0% (~P32)   |

GTC, generalized tonic clonic seizures; −, not observed; ND, not determined; P, postnatal day; +, wild-type allele; fl, floxed allele; d, deleted allele.

*Ella-Cre was removed in Scn1a<sup>B</sup> and Scn1a<sup>B</sup> mice.*
expression in neocortical layer V excitatory neurons, consistent
with previous studies (18,19), and thalamo-cortical projection
neurons. As discussed, our results with VGAT- and Emx1-
double-Cre mice suggest that Nav1.1 haploinsufficiency in exci-
tatory pyramidal cells may have an ameliorating effect on sei-
zuers. Given that impaired function of thalamo-cortical projection neurons has a protective influence on seizure path-
ology (57), Nav1.1 deletion in thalamo-cortical PV projection neurons may contribute to amelioration of epileptic seizures and seizure-related lethality in Scn1a<sup>fl/fl</sup>, PV-Cre-TG mice. This possible ameliorating effect of Nav1.1 deletion in thalamo-
cortical PV projection neurons can also contribute to the better sur-
vival rate in Scn1a<sup>A/+</sup> mice than in Scn1a<sup>A</sup>, Emx1-Cre,
VGAT-Cre mice, in which Nav1.1 expression in thalamo-
cortical PV projection neurons should remain intact. Third, as suggested
by Dutton et al. (18), PV-negative interneurons, such as
somatostatin-, calretinin-, reelin- or neuropeptide Y-positive
cells, may express Nav1.1 (18), and Nav1.1 haploinsufficiency
in these cells may aggravate epileptic phenotypes in Scn1a<sup>B</sup>-
+, VGAT-Cre mice. A caveat to this is that we did not detect
Nav1.1 expression in PV-negative interneurons by immunohis-
tochemistry. Further studies are required to determine the role of
PV-negative interneurons in Dravet syndrome mouse models
and patients.

It has been known that genetic backgrounds largely affect
seizure severity in the mice with Nav1.1 haploinsufficiency
(14,15). In our previous study (15), the mixed C57BL6/129
(75%/25%) background resulted in 25% lethality at 1 week of
age and 40% at 3 weeks, whereas the 129 dominant mixed back-
ground (C57BL6/129 = 25%/75%) did not lead to premature le-
thality. In this study, because all of the conditional knock-out
mouse lines have similar levels of C57BL6/129 (Table 1), con-
tribution of genetic backgrounds to the phenotypic differences
among the conditional lines seems to be minimal.

In summary, the present study showed, for the first time, that
Nav1.1 haploinsufficiencies in excitatory and inhibitory neurons
are both involved in the pathology of Dravet syndrome. Al-
though therapeutic approaches to compensate for Nav1.1 hap-
loinsufficiency are potential treatments for Dravet syndrome,
our results highlight the need for preferential targeting of therapeu-
tic approaches to inhibitory, rather than excitatory neurons.
We also show that a minimal amount of Nav1.1 deletion in PV
cells is enough to cause spontaneous epileptic seizures in
mouse, supporting our previous proposal that functional impair-
ments of PV interneurons are the circuit basis for the epileptic
pathology of Dravet syndrome, and further proposing PV inter-
neurons as promising therapeutic targets.

**MATERIALS AND METHODS**

**Gene targeting and mice**

Mice were handled in accordance with the guidelines of the
Animal Experiment Committee of RIKEN Brain Science Insti-
tute and National Institute of Genetics.

The P1-derived artificial chromosome (PAC) clones (460H9, 5697 and 306M11) were isolated by screening a pooled mouse
genomic PAC library (BACPAC Resource Center, Oakland,
CA, USA) by dot blot hybridization using the probe correspond-
ing to the genomic fragment containing the Scn1a coding exon.
7. A 10.1 kb *EcoRI* fragment of a PAC clone was subcloned into the *EcoRI* site of plBluescript II SK(−) (Agilent Technologies, Santa Clara, CA, USA) to obtain pE8. A 2.6 kb *ApaI* and blunt-ended *EcoT22I* fragment from pE8 was inserted into *ApaI* and blunt-ended *SalI* sites of ploxPrtPKneoRt to generate pL1. pL1 was then digested with *ClaI*, filled with T4 DNA polymerase and the loxP cassette inserted to yield pl2. pE8 was also digested with *SalI* and *AfII*, filled with T4 DNA polymerase and self-ligated to generate pl3. A 6.8 kb *ApaI* fragment of pl3 was inserted into the *ApaI* site of pl2 to yield pl4. In order to generate pP8, a 4.6 kb *PstI* fragment of a PAC clone was subcloned into the *PstI* site of plBluescript II SK(−) (Agilent Technologies). A 3.1 kb *PstI* and blunt-ended *EcoT22I* fragment of pP8 was inserted into *PstI* and blunt-ended *SalI* sites of pEGFP-C2 (Takara Bio, Shiga, Japan) to obtain pR1. A *XhoI* fragment of pMCDTApA (a generous gift from Dr Yagi, Osaka University) was then inserted into an *SalI* site of plR1 to yield pR2. The targeting vector was generated by inserting the *EagI* and *SacI* fragment of pR2 into *NotI* and *SacI* sites of pl4.

The targeting vector was linearized with *SacI* and transfected into 129P2/Ola-derived embryonic stem (ES) cells using a Gene-Pulser (Bio-Rad, Hercules, CA, USA) at 3 μF and 800 V. Transfected ES cells were plated on neomycin-resistant, mitomycin C-treated mouse embryonic feeder cells (MEF). One day after plating, positive selection was performed in the presence of 150 μg/ml of Geneticin (G418; Life Technologies, Carlsbad, CA, USA). Resistant clones were picked at day 7, subsequently expanded into 24-well plates pre-seeded with MEF, and screened by Southern blotting (using both 5′ and 3′ probes located outside the targeting construct) and PCR analysis. Cells from three independent positive clones (1C12, 6A8 and 6G10) were injected into C57BL/6J blastocyst, which were transferred into pseudopregnant female recipient ICR mice. Each ES clone produced male chimeras with >50% agouti coat color, which were bred to C57BL/6J females to allow for the detection of germline transmission. F1 mice heterozygous for the targeted allele were obtained and crossed with *CAG-FLPe* deleter mouse on a C57BL/6J background (58) to obtain N2 mice heterozygous (*Scn1a* +/−, *CAG-FLPe*) for the floxed allele and lacking the neomycin cassette. Absence of the neo-cassette was verified by PCR analysis. N2 *Scn1a* +/−, *CAG-FLPe* mice were subsequently crossed with C57BL/6J mice to obtain N3 *Scn1a* −/+ mice without the *CAG-FLPe* transgene. Absence of the *CAG-FLPe* transgene was verified by PCR analysis. *Scn1a* −/+ mice were thereafter maintained by crossing with C57BL/6J mice. Homozygous (*Scn1a* −/−) mice were obtained by interbreeding N3 or N4 *Scn1a* −/− mice. No phenotypic differences were observed among the mice derived from the three ES cell clones.

To generate constitutive *Scn1a* knock-out mice, *Scn1a* +/− mice were cross-mated with *EIIA*-Cre mice maintained on a C57BL/6J background (22). Heterozygous (*Scn1a* +/−, *EIIA*-Cre) offspring were subsequently backcrossed with C57BL/6J mice to obtain *Scn1a* −/+ mice lacking the *EIIA*-Cre transgene. Absence of the *EIIA*-Cre transgene was verified by PCR analysis. Homozygous (*Scn1a* −/−) mice were obtained by interbreeding *Scn1a* −/− mice.

VGAT (also known as vesicular inhibitory amino acid transporter, Viat)-Cre BAC transgenic mice were generated using a BAC Tg construct made with the Red/ET recombination system (Gene Bridges, GmbH, Dresden, Germany), as previously described (59). Briefly, the Cre-Km cassette, containing Cre recombinase followed by SV40 poly(A) and kanamycin-resistant genes, was PCR-amplified using DNA polymerase Pyrobest (Takara Bio) and the primer set: 5′-GCC TCG CTT CTC TGA CGC GCC CCC GCC GCG TCC CCA GAC CTT TCT TTC TCT CGA CCT GCA GCC CAA GCT GTA CC-3′ and 5′-GGC CTG CGA CTT GTT GCA CAG GGA GTT GCC CAC TAT GGT CAG CTT GCG TCC GAG CAC TAA ATA CAT TCA AAT ATG TAT CC-3′. An *Escherichia coli* strain, containing an RP23-392-P11 BAC clone derived from C57BL/6J mouse genomic DNA (Roswell Park Center Institute, Buffalo, NY, USA) and covering 124 kb upstream and 72 kb downstream of the translational initiation site of the *Vgat* gene, was transformed with pSC101-BAD-gbaA (Gene Bridges) and subsequently with purified PCR products. The resulting BAC Tg construct was verified by restriction enzyme digestion and DNA sequencing. The construct was linearized by PI-SceI and purified using Sepharose columns. The fraction containing the Tg construct was identified by pulsed-field gene electrophoresis (FIBE Mapper, Bio-Rad). The purified DNA fragment was injected into pronuclei of fertilized eggs from C57BL/6J mice to generate Tg mice. Tg65, the Tg mouse line used in this study, has been maintained on a C57BL/6 genetic background. Heterozygous (*Scn1a* +/−, *VGAT*-Cre) mice were obtained by cross-mating *Scn1a* +/−, *Emx1*-Cre mice and *VGAT*-Cre mice. Homozygous (*Scn1a* −/−, *VGAT*-Cre) mice were generated as described below.

*Emx1*-Cre KI mice without a neomycin cassette were described previously (26,60,61), and maintained on a C57BL/6J background. Heterozygous (*Scn1a* +/−, *Emx1*-Cre) mice were obtained by cross-mating *Scn1a* +/−, *Emx1*-Cre mice, and subsequently backcrossed with *Scn1a* +/−, *Emx1*-Cre mice to obtain homozygous (*Scn1a* −/−, *Emx1*-Cre) mice. *Scn1a* −/−, *Emx1*-Cre mice were then cross-mated with *VGAT*-Cre mice to obtain *Scn1a* −/−, *Emx1*-Cre, *VGAT*-Cre mice.

In order to obtain *Scn1a* −/−, *VGAT*-Cre mice, we cross-mated *Scn1a* −/−, *Emx1*-Cre, *VGAT*-Cre mice and *Scn1a* +/−, *VGAT*-Cre mice. However, these crosses yielded only 1 (1 of 96, from 13 litters) *Scn1a* +/−, *VGAT*-Cre mouse, suggesting that the floxed *Scn1a* and *VGAT*-Cre transgene alleles in *Scn1a* +/+, *Emx1*-Cre, *VGAT*-Cre progenitors were mapped close together on the same chromosome and arranged in trans-configuration. To obtain *Scn1a* −/+, *Emx1*-Cre, *VGAT*-Cre offspring in which the floxed *Scn1a* and *VGAT*-Cre transgene alleles were located in cis-configuration, *Scn1a* −/+, *Emx1*-Cre, *VGAT*-Cre progenitors were bred with *Emx1*-Cre or C57BL/6J mice. Triple-heterozygous offspring were subsequently crossed with *Scn1a* −/−, *Emx1*-Cre, *VGAT*-Cre mice to obtain *Scn1a* −/−, *VGAT*-Cre mice, whereas *Scn1a* −/− offspring were crossed with *Scn1a* +/− mice to obtain *Scn1a* −/− controls.

*Pvalb*-Cre transgenic (PV-Cre-TG) mice express Cre in the majority of PV cells—previously described (42), and maintained on a C57BL/6J background. Heterozygous (*Scn1a* +/−, *PV-Cre-TG*) mice were obtained by cross-mating *Scn1a* +/− mice and *PV-Cre-TG* mice. Heterozygous (*Scn1a* +/−, *PV-Cre-TG*) mice were obtained by backcrossing *Scn1a* +/−, *PV-Cre-TG* mice with *Scn1a* +/− mice. Crosses between *Scn1a* +/−, *PV-Cre-TG* females and *Scn1a* +/− males produced *Scn1a* +/−, *PV-Cre-TG* and *Scn1a* +/− mice, in addition to *Scn1a* +/−.
PV-Cre-TG, Scn1a<sup>B±</sup>, PV-Cre-TG and Scn1a<sup>B+</sup> mice. This suggests that PV-Cre-TG-mediated recombination occurred in female germ line cells.

Pvalb-IREs-Cre knock-in (PV-Cre-KI) mice, with an IRES-Cre cassette inserted into the 3′ untranslated region of the mouse Pvalb allele through gene targeting, were described previously (48). PV-Cre-KI mice express Cre only in PV cells (49–52). PV-Cre-KI mice were obtained on a C57BL/6J background. Heterozygous (Scn1a<sup>B±</sup>, PV-Cre-KI) mice were obtained by cross-mating Scn1a<sup>B±</sup> mice and PV-Cre-KI mice and subsequently backcrossed with Scn1a<sup>B±</sup> mice to obtain homozygous (Scn1a<sup>B±</sup>, PV-Cre-KI) mice.

A Rosa26-LacZ transgenic reporter mouse strain for Cre-mediated recombination was described previously (25). The LacZ coding sequence was fused to a nuclear localization signal. A Gad6<sup>77-GFP</sup> mouse strain for the labeling of inhibitory neurons was described previously (29).

Electrocorticographic recordings

Stainless steel screws (1.1 mm diameter) served as electrocorticographic electrodes and were implanted over the somatosensory cortex (1.5 mm lateral to midline, 1.0 mm posterior to bregma) under 1.5% halothane anesthesia with N<sub>2</sub>O:O<sub>2</sub> (3:2) ventilation. A ground electrode was implanted on the cerebellum (at midline, 2.0 mm posterior to lambda). Electromyogram (EMG) electrodes were also placed in the cervical region of the trapezius. Low-amplitude fast ECoG activity, together with high levels of EMG activity, indicates that the mouse was awake. Large-amplitude slow ECoG activity with low levels of EMG activity indicates the mouse was in a resting or sleeping state.

Western blot analysis

Brains were isolated from mice and homogenized in homogenization buffer [0.32 m sucrose, 10 mM HEPES, 2 mM EDTA and 1× complete protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA), pH 7.4]. Homogenates were centrifuged for 15 min at 1000 g to separately yield the supernatant and pellets. Homogenates from 5–10 mice were subsequently mixed background from the Jackson laboratory (JAX#008069; Bar Harbor, ME, USA) and maintained on a C57BL/6J background. Heterozygous (Scn1a<sup>B±</sup>, PV-Cre-KI) mice were obtained by cross-mating Scn1a<sup>B±</sup> mice and PV-Cre-KI mice and subsequently backcrossed with Scn1a<sup>B±</sup> mice to obtain homozygous (Scn1a<sup>B±</sup>, PV-Cre-KI) mice.

Immunohistochemistry and immunofluorescence histochemistry

Mice were deeply anesthetized and perfused transcardially with 4% paraformaldehyde in PBS (10 mM phosphate buffer, 2.7 mM KCl and 137 mM NaCl, pH 7.4). Brains were removed from the skull, postfixed in 4% paraformaldehyde in PBS for 24 or 4 h (only for X-gal staining) at 4°C and cryoprotected in 30% sucrose in PBS. Free-floating sections of mouse brains (30 μm) were incubated in 10 mM citric acid (pH 6.0) and 1 mM EDTA (pH 8.0) at 100°C for 20 min, blocked with 4% Block Ace (DS Pharma Biomedical, Osaka, Japan) in PBS for 1 h at room temperature and incubated with rabbit anti-C-terminal Nav1.1 antibody (IO1; 250 ng/ml; 15) for 3 overnight (50 h) at room temperature. Endogenous peroxidases were quenched by incubation with 0.3% hydrogen peroxide in PBS. The sections were then incubated with biotinylated goat or donkey polyclonal secondary antibody (1:200; Vector, Burlingame, CA, USA). Detection of antibody–antigen complexes was performed using Vectastain Elite ABC (Vector) and Nova Red (Vector) kits. Sections were then mounted flat on glass slides.

Immunofluorescence histochemistry was performed using rabbit anti-GABA (A2052; 1:4000; Sigma-Aldrich) and chicken anti-β-galactosidase (ab9361; 1:10000; Abcam) antibodies. Secondary antibodies were Alexa Fluor 488-, 594- and 647-conjugated (1:1000; Life Technologies). Images were obtained with a TCS SP2 microscope (Leica Microsystems, Wetzlar, Germany) and processed using Adobe Photoshop Elements 4.0 (Adobe Systems, San Jose, CA, USA). Brain sections mounted on MAS-coated slide glasses (Matsunami S9441) (10 μm) or floating (40 μm) were also stained with LacZ substrate solution (1 mg/ml X-gal, 2 mM MgCl<sub>2</sub>, 5 mM K<sub>3</sub>Fe(CN)<sub>6</sub>, 5 mM K<sub>4</sub>Fe(CN)<sub>6</sub>/PBS overnight at 37°C and subsequently incubated with an antibody against GABA (1:2000) at 4°C overnight. After incubation in biotinylated goat anti-rabbit IgG (1:200) at room temperature for 1 h, sections were incubated in Vectastain Elite ABC Kit (PK-6101; Vector) for 1 h at room temperature. Signals were detected by 5 min DAB treatment.

For immunohistochemistry double-staining on Gad6<sup>77-GFP</sup> mice (29), mice were perfused transcardially with peridate-lysine-4% paraformaldehyde (PLP, 10 mM NaO<sub>4</sub>, 75 mM lysine, 37.5 mM phosphate buffer, with 4% paraformaldehyde). Brains were removed, postfixed in PLP solution overnight at 4°C and paraffin-embedded. Paraffin-embedded mouse brains were sectioned (6 μm), incubated for 12 h at 48°C and stored at room temperature. Sections were then deparaffinized, rehydrated and microwaved in 1 mM EDTA, pH 8.0. After blocking slides with 4% BlockAce solution in PBS containing 0.05% Tween 20, sections were incubated with goat anti-internal-region Nav1.1 antibody (C-18; 1:500; Santa Cruz Biotechnology) and a mixture of monoclonal anti-GFP antibodies (1:500; Roche

Health, Bethesda, MD, USA). Mean expression levels were estimated by comparison with serial dilutions of homogenates from age-matched control mice and represented as percentages relative to control mice. Statistical comparisons were made using Student’s t-tests. Values with P < 0.05 were considered significant. At least two independent assays were performed.
REFERENCES

8. Nakayama, T., Ogihara, I., Ito, K., Kaneda, M., Mazaki, E., Osaka, H., Ohtani, H., Inoue, Y., Fujisawa, T., Uematsu, M. et al. (2010) Deletions of SCN1A 5′ genomic region with promoter activity in Dravet syndrome. *Hum. Mut.*, 31, 820–829.

9. Engel, J. Jr (2001) A proposed diagnostic scheme for people with epileptic seizures and with epilepsy: report of the ILAE Task Force on Classification and Terminology. *Epilepsia*, 42, 796–803.

10. Dravet, C., Bureau, M., Oguni, H., Fukushima, Y. and Cokar, O. (2005) Severe myoclonic epilepsy in infancy (Dravet syndrome). In Roger, J., Bureau, M., Dravet, C., Genton, P., Tassinari, C.A. and Wolf, P. (eds), *Epileptic Syndromes in Infancy, Childhood and Adolescence*, 4th edn. John Libbey Eurotext Ltd, Montrouge, France, pp. 89–113.

11. Sugawara, T., Tsurubuchi, Y., Fujisawa, T., Mazaki-Miyazaki, E., Nagata, K., Montal, M., Inoue, Y. and Yamakawa, K. (2003) Nav1.1 channels with mutations of severe myoclonic epilepsy in infancy display attenuated currents. *Epilepsy Res.*, 54, 201–207.

12. Meisler, M.H. and Kearney, J.A. (2005) Sodium channel mutations in epilepsy and other neurological disorders. *J. Clin. Invest.*, 115, 2010–2017.

13. Mulley, J.C., Scheffer, I.E., Petrou, S., Dibbens, L.M., Berkovic, S.F. and Harkin, L.A. (2005) SCN1A mutations and epilepsy. *Hum. Mutat.*, 25, 535–542.

14. Yu, F.H., Mantegazza, M., Westenbroek, R.E., Robbins, C.A., Kalume, F., Burton, K.A., Spain, W.J., McKindry, G.S., Scheuer, T. and Catterall, W.A. (2006) Reduced sodium current in GABAergic interneurons in a mouse model of severe myoclonic epilepsy. *Nat. Neurosci.*, 9, 1142–1149.

15. Ogihara, I., Miyamoto, H., Morita, N., Apotrou, N., Mazaki, E., Inoue, I., Takeuchi, T., Itohara, S., Yamagawa, Y., Obata, K. et al. (2007) Na1.1 localizes to axons of parvalbumin-positive inhibitory interneurons: a circuit basis for epileptic seizures in mice carrying an SCN1A gene mutation. *J. Neurosci.*, 27, 5903–5914.

16. Han, S., Tai, C., Westenbroek, R.E., Yu, F.H., Cheah, C.S., Potter, G.B., Rubenstein, J.L., Scheuer, T., de la Iglesia, H.O. and Catterall, W.A. (2012) Autistic-like behaviour in *Scn1a*−/− mice and rescue by enhanced GABA-mediated neurotransmission. *Nature*, 489, 385–390.

17. Ito, S., Ogihara, I., Yamada, K., Miyamoto, H., Hensch, T.K., Osawa, M. and Yamakawa, K. (2013) Mouse with Nav1.1 haploinsufficiency, a model for Dravet syndrome, exhibits lowered sociability and learning impairment. *Neurobiol. Dis.*, 49, 29–41.

18. Dutton, S.B., Makond, C.D., Papale, L.A., Shankar, A., Balakrishnan, B., Nakazawa, K. and Escayg, A. (2013) Preferential inactivation of *Scn1a* in parvalbumin interneurons increases seizure susceptibility. *Neurobiol. Dis.*, 49, 211–220.

19. Cheah, C.S., Yu, F.H., Westenbroek, R.E., Kalume, F.K., Oakley, J.C., Potter, G.B., Rubenstein, J.L. and Catterall, W.A. (2012) Specific deletion of NaV1.1 sodium channels in inhibitory interneurons causes seizures and premature death in a mouse model of Dravet syndrome. *Proc. Natl Acad. Sci. USA*, 109, 14646–14651.

20. Potter, G.B., Petryniak, M.A., Shevchenko, E., McKinsey, G.L., Ekkcr, M. and Rubenstein, J.L. (2009) Generation of Cre-transgenic mice using Dla1/Dla2 enhancers and their characterization in GABAergic interneurons. *Mol. Cell. Neurosci.*, 40, 167–186.

21. Belforte, J.E., Zsivos, V., Sklar, E.R., Jiang, Z., Yu, G., Li, Y., Quinlan, E.M. and Nakazawa, K. (2010) Postnatal NMDA receptor ablation in corticocortical interneurons confers schizophrenia-like phenotypes. *Nat. Neurosci.*, 13, 76–83.

22. Laks, M., Pichel, J.G., Gorman, J.R., Sauer, B., Okamoto, Y., Lee, E., Alt, F.W. and Westphal, H. (1996) Efficient in vivo manipulation of mouse *Scn1a* gene mutations associated with Dravet syndrome. *Epilepsy Res.*, 28, 49–115.

23. Chaudhry, F.A., Reimer, R.J., Bellocchio, E.E., Danbolt, N.C., Osen, K.K., Belforte, J.E., Zsivos, V., Sklar, E.R., Jiang, Z., Yu, G., Li, Y., Quinlan, E.M. and Nakazawa, K. (2010) Postnatal NMDA receptor ablation in corticocortical interneurons confers schizophrenia-like phenotypes. *Nat. Neurosci.*, 13, 76–83.

24. Wojcik, S.M., Katsurabayashi, S., Guillemin, I., Friauf, E., Rosenmund, C., Cho, S., Takemori, N., Shimizu, S., Fujii, T., Nakazawa, K. and Carvey, P.M. (2007) Cortical adenyl cyclase 1 is required for thalamocortical synapse maturation and aspects of layer IV barrel development. *J. Neurosci.*, 28, 5931–5943.
40. Petreanu, L., Mao, T., Sternson, S.M. and Svoboda, K. (2009) The
41. Feldmeyer, D., Lübke, J. and Sakmann, B. (2006) Efficacy and connectivity
42. Herkenham, M. (1980) Laminar organization of thalamic projections to the
43. Tanahira, C., Higo, S., Watanabe, K., Tomioka, R., Ebihara, S., Kaneko, T.
44. de Lecea, L., del Río, J.A. and Soriano, E. (1995) Developmental expression
45. de Lecea, L., del Río, J.A. and Soriano, E. (1995) Developmental expression
46. Bishop, G.A. (1993) An analysis of HRP-filled basket cell axons in the cat’s
cerebellum. I. Morphometry and configuration. Anat. Embryol. (Berl.), 188,
287–297.
47. Sugihara, I., Fujita, H., Na, J., Quy, P.N., Li, B.Y. and Ikeda, D. (2009)
48. Hippenmeyer, S., Vrieseling, E., Sigrist, M., Portmann, T., Laengle, C.,
Ladle, D.R. and Arber, S. (2005) A developmental switch in the response of
DRG neurons to ETS transcription factor signaling. PLoS Biol., 3, e159.
49. Madisen, L., Zwingman, T.A., Sunkin, S.M., Oh, S.W., Zariwala, H.A., Gu,
H., Ng, L.L., Palmer, R.D., Hawrylycz, M.J., Jones, A.R. et al. (2010) A
50. Wen, L., Lu, Y.S., Zhi, X.H., Li, X.M., Woo, R.S., Chen, Y.J., Yin, D.M.,
Lai, C., Terry, A.V. Jr, Vazdarjanova, A. et al. (2010) Neuregulin 1 regulates
pyramidal neuron activity via ErbB4 in parvalbumin-positive interneurons.
51. Atallah, B.V., Bruns, W., Carandini, M. and Scanziani, M. (2012)
Parvalbumin-expressing interneurons linearly transform cortical responses to
visual stimuli. Neuron, 73, 159–170.
52. Shamir, A., Kwon, O.B., Karavanoa, I., Vullhorst, D., Leiva-Salcedo, E.,
Janssen, M.J. and Buonanno, A. (2012) The importance of the NRG-1/ErbB4
pathway for synaptic plasticity and behaviors associated with psychiatric
disorders. J. Neurosci., 32, 2988–2997.
53. Freund, T.F. and Buzsáki, G. (1996) Interneurons of the hippocampus.
54. Klausberger, T., Magill, P.J., Márton, L.F., Roberts, J.D., Cobden, P.M.,
Buzsáki, G. and Somogyi, P. (2003) Brain-state- and cell-type-specific firing of
hippocampal interneurons in vivo. Nature, 421, 844–848.
55. Gulyás, A.I., Szabó, G.G., Ulbert, I., Holderith, N., Monyer, H., Erdélyi, F.,
Szabó, G., Freund, T.F. and Hájos, N. (2010) Parvalbumin-containing fast-spiking basket cells generate the field potential oscillations induced by
cholinergic receptor activation in the hippocampus. J. Neurosci., 30, 15134–
15145.
56. Celio, M.R. (1990) Calbindin-D-28k and parvalbumin in the rat nervous system.
Science, 35, 375–475.
57. Paz, J.T., Davidson, T.J., Frechette, E.S., Delord, B., Parada, I., Peng, K.,
Deisseroth, K. and Huguenard, J.R. (2013) Closed-loop optogenetic control of
thalamus as a new tool to interrupt seizures after cortical injury. Nat. Neurosci.,
16, 64–70.
58. Iwashita, T., Katah, H., Nishimaru, H., Ishikawa, Y., Inoue, H., Saito, Y.M.,
Ando, R., Iwama, M., Takahashi, R., Negishi, M. et al. (2007) Rac-GAP
alpha-chimerin regulates motor-circuit formation as a key mediator of
EphrinB3/EphA4 forward signaling. J. Neurosci., 37, 137–141.
59. Iwashita, T., Nomura, R., Ando, R., Ikeda, T., Tanaka, M. and Itohara, S.
(2004) Dorsal telencephalon-specific expression of Cre recombine in PAs
transgenic mice. Genesis, 38, 130–138.
60. Iwashita, T., Datwani, A., Wolf, A.M., Nishiyama, H., Taguchi, Y.,
Tonegawa, S., Knöpfel, T., Erzurumlu, R.S. and Itohara, S. (2000) Cortex-restricted disruption of NMDAR1 impairs neuronal patterns in the
barrel cortex. Nature, 406, 726–731.