genesis 45:547–553 (2007)

TECHNOLOGY REPORT

Floxed Allele for Conditional Inactivation of the Voltage-Gated Sodium Channel β1 Subunit *Scn1b*

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Received 1 June 2007; Accepted 25 June 2007

Summary: The voltage-gated sodium channel gene *Scn1b* encodes the auxiliary subunit β1, which is widely distributed in neurons and glia of the central and peripheral nervous systems, cardiac myocytes, skeletal muscle myocytes, and neuroendocrine cells. We showed previously that the *Scn1b* null mutation results in a complex and severe phenotype that includes retarded growth, seizures, ataxia, and death by postnatal day 21. We generated a floxed allele of *Scn1b* by inserting loxP sites surrounding the second coding exon. Ubiquitous deletion of the floxed exon by Cre recombinase using CMV-Cre-transgenic mice produced the *Scn1b*^{del} allele. The null phenotype of *Scn1b*^{del} homozygotes is indistinguishable from that of *Scn1b* nulls and confirms the in vivo inactivation of *Scn1b*. Conditional inactivation of the floxed allele will make it possible to circumvent the lethality that results from complete loss of this gene, such that the physiological role of *Scn1b* in specific cell types and/or specific developmental time points can be investigated. genesis 45:547–553, 2007. © 2007 Wiley-Liss, Inc.

Key words: *Scn1b*; β1; conditional allele; Cre/loxP

Voltage-gated sodium channels initiate and propagate action potentials in excitable cells and are key modulators of electrical signaling (Catterall, 2000). Sodium channels are composed of a central, pore-forming α subunit and two auxiliary β subunits. The β subunits do not form the pore but play important roles in channel gating, channel cell surface expression, and cell-to-cell communication (Isom, 2002; Meadows and Isom, 2005). Sodium channels are unique among voltage- and ligand-gated ion channels in that their auxiliary β subunits both modulate channel function and serve as cell adhesion molecules (CAMs) through their extracellular immunoglobulin (Ig)-like domains. We and others have demonstrated that β subunits, especially β1 (Scn1b), function as CAMs: Scn1b interacts with the extracellular matrix protein tenascin-R to influence cell migration (Xiao *et al.*, 1999); Scn1b participates in homophilic cell adhesion, resulting in cellular aggregation and ankyrin recruitment (Malhotra *et al.*, 2000, 2002); Scn1b interacts heterophilically with the CAMs contactin, neurofascin-155, neurofascin-186, and NrCAM (Kazarinova-Noyes *et al.*, 2001; McEwen and Isom, 2004; McEwen *et al.*, 2004; Ratcliffe *et al.*, 2001); Scn1b interacts with receptor phosphotyrosine phosphatase β (Ratcliffe *et al.*, 2000); Scn1b interactions with contactin and neurofascin-186 result in increased channel cell surface expression (Kazarinova-Noyes *et al.*, 2001; McEwen and Isom, 2004; McEwen *et al.*, 2004); and Scn1b promotes neurite extension as a result of homophilic adhesion (Davis *et al.*, 2004). Mutations in the extracellular Ig domain of Scn1b are linked to generalized epilepsy with febrile seizures plus (GEFS+1) and/or temporal lobe epilepsy in human patients (Audenaert *et al.*, 2003; Scheffer *et al.*, 2006; Wallace *et al.*, 1998, 2002). Thus, we propose that, as a channel modulator, Scn1b plays important roles in the control of electrical signaling and, as a CAM, Scn1b acts as a critical communication link between extra- and intracellular signaling molecules.

β1 is encoded by a single gene in the mammalian genome, *SCN1B* (Makita *et al.*, 1994a), that is expressed in central and peripheral neurons (Altman, 1972a,b; Sashihara *et al.*, 1995; Singh, 1977; Sutkowski and Catterall, 1990), including nodes of Ranvier in myelinated axons (Chen *et al.*, 2004), glia (Blackburn-Munro and Fleetwood-Walker, 1999; Oh and Waxman, 1994, 1995; Oh *et al.*, 1995, 1997), cardiac and skeletal muscle myocytes (Maier *et al.*, 2004; Makita *et al.*, 1994a; Malhotra *et al.*, 2001), and neuroendocrine cells (Kazen-Gillespie *et al.*, 2000). These data suggested that complete loss of

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Contract grant sponsor: National Multiple Sclerosis Society, Contract grant number: RG2882, Contract grant sponsor: NIH, Contract grant number: R01 MH059980
Published online 14 September 2007 in
Wiley InterScience (www.interscience.wiley.com).
DOI: 10.1002/dvg.20324

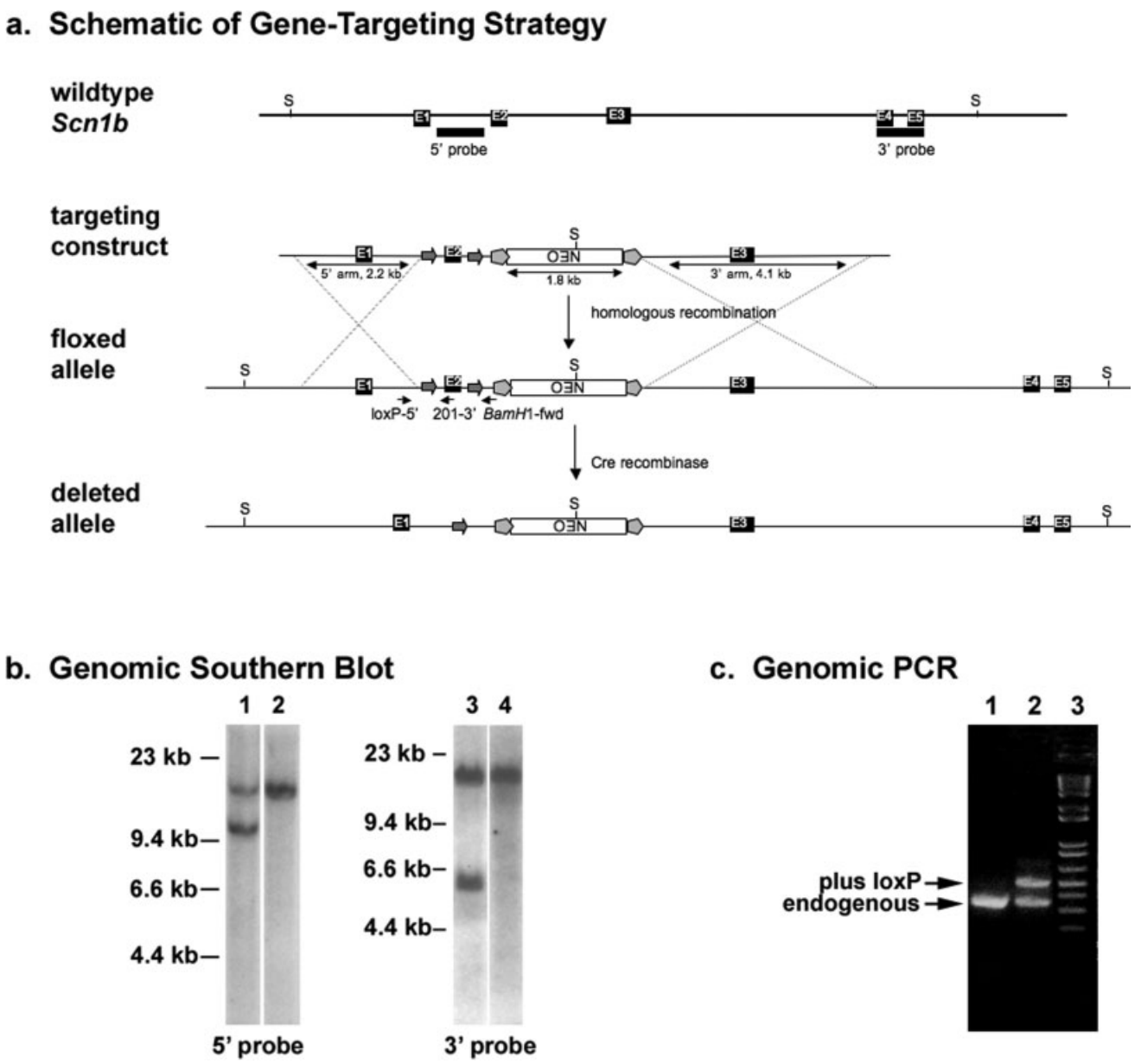

FIG. 1. Targeting of *Scn1b*. (**a**) Schematic of gene-targeting strategy: The targeting construct contained exons 1, 2, and 3 (E1–E3), with E2 flanked by loxP sites (arrows) and an inverted PGK-neocassette flanked by FRT sites (pentagons). There was 2.2 kb of homologous sequence in the left (5′) arm and 4.1 kb in the right (3′) arm. The 5′ and 3′ Southern probes, as well as diagnostic Spe1 (S) restriction sites are indicated. (**b**) Southern blots of ES cell genomic DNA digested with SpeI. Lanes 1 and 2: blot probed with 5′ probe shown in panel a. Lane 1, correctly targeted. Lane 2, wild type. Lanes 3 and 4: blot probed with 3′ probe shown in panel a. Lane 3, correctly targeted. Lane 4, wild type. (**c**) PCR of ES cell genomic DNA showing correct targeting of the left (5′) loxP site. Lower band (310 bp), endogenous allele. Upper band (430 bp), correctly targeted allele.

SCN1B in vivo would produce a complex and severe phenotype. In support of this, we showed that *Scn1b* null mice are ataxic, display spontaneous generalized seizures, are growth retarded, and die by 3 weeks of age (Chen *et al.*, 2004). To understand the physiological role of *Scn1b* in normal development as well as in brain and heart disease, and to circumvent the lethality associated with complete loss of this gene, we generated mice carrying the conditional $Scn1b^{flox}$ allele.

Scn1b is composed of five coding exons spanning ~10 kb of genomic DNA (Makita *et al.*, 1994b). We introduced loxP sites at positions 190 bp upstream and 220 bp downstream, respectively, of exon 2 in *Scn1b* cloned from a mouse 129 library, as described previously (Chen *et al.*, 2004; Fig. 1). Exon 2 was chosen over exon 3 for targeting [as in our previous targeting experiment (Chen *et al.*, 2004)] so as to leave intron 3 intact for expression of the splice variant β1A, retaining this

intron, in the targeted mice prior to Cre recombinase expression (Kazen-Gillespie *et al.*, 2000). Exon 2 of *Scn1b* contains the amino acid sequence: GAVLVSSAWG*G*CV EVDSETEAVYGMTFKIL*C*ISCKRRSETTAETFTEWTFRQKG TEEFVK, that includes the amino-terminus of the mature Scn1b protein after removal of the signal sequence (*G*) as well as the first cysteine residue of the Ig loop domain (*C*). Thus, following deletion of this exon, we predicted that it would be impossible for functional Scn1b protein to be expressed. The floxed exon and two homology arms of 2.2 and 4.1 kb each, containing exons 1 and 3, respectively, were assembled in a vector containing a neocassette flanked by FRT sites (Fig. 1a). The FRT sites were included to permit in vivo deletion of the neocassette if its presence was found to interfere with the expression of the targeted allele (Dymecki, 1996).

The targeting vector was electroporated into PAT-5 embryonic stem (ES) cells (Chen *et al.*, 2004; Domino *et al.*, 2001) and 480 neomycin-resistant colonies were recovered. These colonies were screened by Southern blot using both a 5′ probe, located within the 5′ homology arm, and a 3′ probe, located downstream of the 3′ homology arm, as indicated in Figure 1a. Eleven of the 480 colonies screened were correctly targeted as defined by Southern blot analysis. Figure 1b shows Southern blots of a correctly targeted ES cell clone. We found that the 5′ loxP site was lost in some clones during targeting and thus screened for retention of this site by PCR in all of the clones determined to be correctly targeted by Southern blot (Fig. 1c). Using these criteria, nine correctly targeted recombinants were obtained, giving a final targeting efficiency of ~2%. These ES cell clones were subjected to chromosome analysis, and ultimately two (1F1 and 5A2) were injected into C57BL/6 blastocysts to produce chimeric mice. Germline transmission was obtained from the 1F1 line but not from the 5A2 line.

Crossing chimeric mice to strain C57BL/6 mice generated $Scn1b^{neo/+}$ mice that were heterozygous for the neoallele shown in Figure 1a. To determine whether the neocassette interfered with *Scn1b* expression, these mice were then bred to homozygosity. In contrast to other conditional null sodium channel gene targeting experiments (Levin and Meisler, 2004), $Scn1b^{flox/flox}$ mice exhibited a normal phenotype, lived normal life spans, and bred normally. These results suggested that neither the neocassette nor the loxP sites present in the targeted allele affected *Scn1b* expression. Thus, we elected to proceed directly to the Cre recombinase breeding step (Fig. 1a) and leave the neocassette intact in the targeted allele.

To determine whether deletion of exon 2 would inactivate *Scn1b*, homozygous $Scn1b^{flox/flox}$ mice were crossed with CMV-Cre-transgenic mice. The predicted deleted allele, shown in Figure 2, was detected in heterozygous offspring ($Scn1b^{del/+}$ mice) by amplification of a 436-bp fragment from genomic DNA using the primers loxP-5′ and BamH1-fwd (Fig. 2a). Intercrosses between heterozygous $Scn1b^{del/+}$ mice generated homozygous mice ($Scn1b^{del/del}$) that exhibited severe seizures beginning at approximately postnatal day 10, retarded growth (Fig. 2b), ataxia, and 100% lethality by 3 weeks of age, a phenotype that was indistinguishable from mice carrying the *Scn1b* null allele (Chen *et al.*, 2004). All of the affected mice were homozygous for the deleted allele (Fig. 2a, del/del).

We performed in situ hybridization analysis to determine the extent of *Scn1b* inactivation in $Scn1b^{del/del}$ brains at P17-18 (Fig. 2c). *Scn1b* mRNA was extensively reduced in all brain areas compared to $Scn1b^{flox/+}$ mice. Western blot analysis showed that levels of Scn1b protein expression in brain membrane preparations from $Scn1b^{flox/+}$ mice were similar to wild-type mice (Fig. 2d). Importantly, $Scn1b^{del/del}$ mice showed no Scn1b protein expression, similar to *Scn1b* null mice (Fig. 2d; Chen *et al.*, 2004), and exhibited an indistinguishable behavioral phenotype. Thus, the behavioral and molecular phenotypes of $Scn1b^{del/del}$ mice demonstrate successful Cre-mediated inactivation of *Scn1b*.

In conclusion, we generated a floxed allele of *Scn1b* that can be inactivated by expression of Cre recombinase. The floxed allele produces normal levels of Scn1b protein and homozygotes, for this allele does not exhibit behavioral or molecular abnormalities. In vivo deletion of exon 2 by Cre recombinase results in complete loss of *Scn1b* expression. Mice carrying the $Scn1b^{flox}$ allele will be critical to the future dissection of the physiological consequences of *Scn1b* inactivation in specific cell types and/or at specific developmental time points, and thus to the understanding of the mechanism of Scn1b function.

MATERIALS AND METHODS

Materials

PAT-5 ES cells were obtained from the University of Michigan Transgenic Animal Model Core Laboratory (Domino *et al.*, 2001). CMV-Cre-transgenic mice were obtained from Dr. Anand Swaroop at the University of Michigan. The plasmid, ploxPFlpneo (Hiraoka *et al.*, 2006), containing two loxP sites and a neocassette flanked by FRT sites, was obtained from the University of Michigan Transgenic Animal Model Core Laboratory. A rabbit polyclonal antibody raised against the C-terminus of Scn1b (Oyama *et al.*, 2006) was obtained from Dr. N. Nukina, Dr. Oyama, and Dr. Miyazaki at the RIKEN Brain Institute.

Construction of the Targeting Vector and Screening of ES Cells

Scn1b was cloned from a mouse genomic 129 library (Stratagene, LaJolla, CA) using the full-length rat *Scn1b* cDNA as a probe, as previously described (Chen *et al.*, 2004). A 2.2-kb genomic fragment containing a 5′ flanking region and exon 1 was inserted into the Kpn1 site of ploxPFlpneo (Hiraoka *et al.*, 2006). A 540-bp fragment containing exon 2 was inserted into the BamH1 site of ploxPFlpneo. Finally, a 4.1-kb fragment containing exon

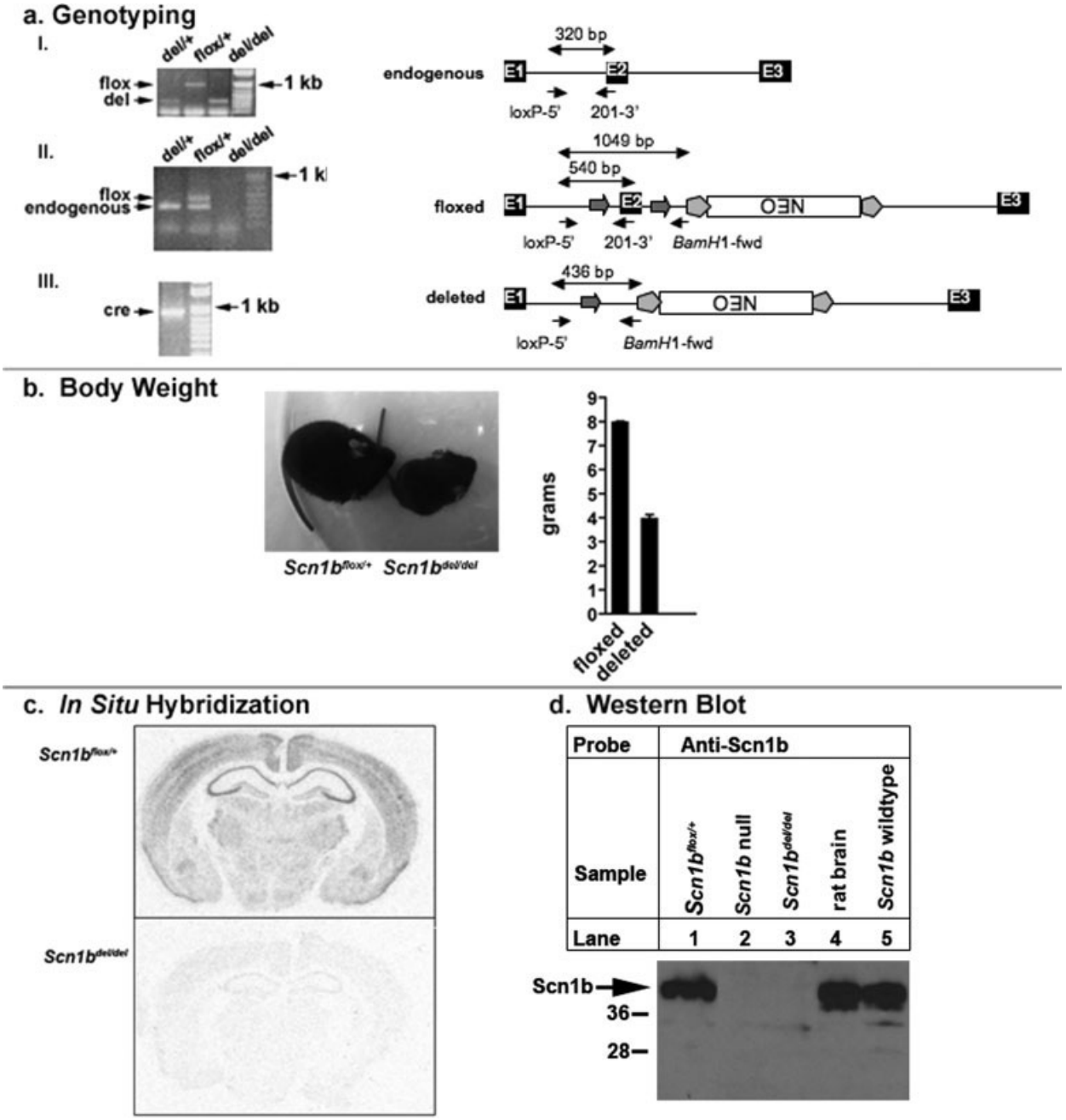

FIG. 2. Mouse characterization. (**a**) Genotyping—Left panel: **I.** PCR of tail DNA using primer pair loxP-5± and BamH1-fwd. The deleted allele (del) generated a 436-bp product and the floxed allele (flox) a 1,049 bp product, as indicated by the arrows. The endogenous allele (+) is not detected with this primer pair. **II.** PCR of genomic DNA using primer pair loxP-5′ and 201-3′. The endogenous allele (+) generated a 320-bp product, and the floxed allele (flox) generated a 540-bp product. The deleted allele (del) is not detected with this primer pair. **III.** PCR of tail DNA using primer pair CreF and CreR to detect Cre recombinase. The Cre allele generated a 850-bp band. Right panel: locations of primers and expected PCR bands from endogenous, floxed, and deleted alleles. (**b**) Body weight: **I:** comparison of typical *Scn1b*$^{flox/+}$ and *Scn1b*$^{del/del}$ littermate mice at P17. **II:** body weights of P16-17 *Scn1b*$^{flox/+}$ vs. *Scn1b*$^{del/del}$ littermate mice. Mean and SEM are presented. For *Scn1b*$^{flox/+}$: mean = 7.95 ± 0.07 g; for *Scn1b*$^{del/del}$: mean = 3.93 ± 0.19 g, $P < 0.0001$. (**c**) In situ hybridization: coronal brain slices from P17 *Scn1b*$^{flox/+}$ and *Scn1b*$^{del/del}$ littermate mice were prepared for in situ hybridization with ^{35}S-labeled *Scn1b* sense and antisense mRNA probes as described in Material and Methods. **I.** *Scn1b*$^{flox/+}$ brain probed with ^{35}S-labeled *Scn1b* antisense probe. **II.** *Scn1b*$^{del/del}$ brain probed with ^{35}S-labeled *Scn1b* antisense probe. ^{35}S-labeled *Scn1b* sense probes gave no signal above background on brain slices from *Scn1b*$^{flox/+}$ brains, similar to the image shown in II (data not shown). (**d**) Western blot: Brain membranes were prepared from P17-18 *Scn1b*$^{flox/+}$, *Scn1b*$^{del/del}$, *Scn1b* wild-type, and *Scn1b* null mice. Western blot analysis was performed on equal aliquots of protein as described in Materials and Methods. Rat brain membranes, that had been previously prepared and stored at −80°C, were used as a positive control. An anti-Scn1b rabbit polyclonal antibody (1:500 dilution, obtained from Dr. N. Nukina (Oyama *et al.*, 2006)) was used to detect Scn1b protein. Arrow indicates migration of wild-type Scn1b subunits. Molecular weights are indicated in kilodaltons.

3 and the 3′ flanking region was inserted into the Swa1 site of ploxPFlpneo. These manipulations resulted in insertion of the FRT-neo-FRT cassette into intron 2 of *Scn1b* in the 3′ to 5′ orientation relative to the *Scn1b* coding sequence as well as the insertion of loxP sites in the introns flanking exon 2. Probes to detect homologous recombination on the 5′ and 3′ arms following digestion of genomic DNA with SpeI were the same as those used in our previous *Scn1b* targeting experiment (Chen *et al.*, 2004). PAT-5 ES cells were electroporated and 480 clones were screened by Southern blot using 5′ and 3′ probes as previously described (Chen *et al.*, 2004). We found that in many clones the 5′ loxP site was lost during targeting. Thus, we also developed a PCR experiment using primers flanking this loxP site (loxP-5′: GTTACTCACCACAGTGACATCCTC, and 201-3′: AGGCTGAGGATACTGCGGACACAGTGGAC) to determine correct targeting. Nine clones were identified as correctly targeted, subjected to chromosome analysis, and two clones (1F1 and 5A2) injected into C57BL/6 blastocysts. High-percentage chimeric animals were obtained from the 1F1 breeding and bred to C57BL/6 mice to produce heterozygous animals. Genotyping was performed by PCR using tail DNA, as described later. ES cell electroporation and colony selection, injection of blastocysts, and generation of chimeric mice were provided as a service of the Transgenic Animal Model Core Laboratory at the University of Michigan. All experiments were carried out in accordance with the guidelines for Animal Care of the University of Michigan.

PCR Analysis

Floxed and deleted mice were genotyped using two sets of primers: a set of primers external to the loxP sites that amplified a 1,049-bp band corresponding to the floxed allele or a 436-bp band corresponding to the deleted allele (Fig. 2a, right panel; loxP-5′: described earlier and BamH1-fwd: 5′-GCGGTGGGCTCTATGGCTTCTGAGG-3′); and a set of primers which amplified a region surrounding the 5′ loxP site (Fig. 2a, right panel; loxP-5′: and 201-3′, described earlier). This second set of primers amplified a 540-bp band from the floxed allele, a 320-bp band from the endogenous allele, and no band from the deleted allele. Cre recombinase was detected by PCR using the primers CreF-2: 5′-TCCAATTTACTGACCGTACACC-3′ and CreR: 5′ GGTATCTCTGACCAGAGTCATC-3′, which amplified a 850-bp band (Fig. 1c).

In Situ Hybridization Analysis

Generation of mRNA probes. A cDNA template for generation of *Scn1b* sense and antisense probes was prepared by RT-PCR using mouse brain total RNA as template and the primers, *Scn1b*-probe-Xho5′ (5′-CAACTCGAGCGCGGCCATGGGGACGCTGCTGG-3′) and *Scn1b*-probe-Kpn3′ (5′-CAAGGTACCAGAGCCAGCGCTATTCAGCCAC-3′), based on the mouse *Scn1b* cDNA sequence NM 011322. The resulting PCR product was subcloned into pBluescript and the correct DNA sequence was confirmed by sequencing. To generate sense probe, the template was linearized with *Kpn1*, and T3 RNA polymerase (Promega, Madison, WI) was used in the labeling reaction. To generate antisense probe, the template was linearized with *Xho1*, and T7 RNA polymerase (Promega) was used in the labeling reaction. ^{35}S-labeled mRNA probes were prepared in the following mixture, incubated for 2 h at 37°C (total volume: 25 μl): 5 μl H_2O, 4 μl ^{35}S-UTP (SJ603, 20 mCi/ml, Amersham, Piscataway, NJ), 2 μl ^{35}S-CTP (SJ40382, 40 mCi/ml, Amersham), 5 μl 5× transcription buffer, 2.5 μl 100 mM dithiothreitol (DTT), 2 μl linearized cDNA (0.5–1 μg), 1 μl 10 mM GTP, 1 μl 10 mM ATP 1 μl RNAse inhibitor (>20 U), 1.5 μl RNA polymerase, as indicated earlier. One microliter of RNAse-free DNAse was then added for 15 min at room temperature. Labeled probes were separated from free isotope by passing over a Micro Bio-spin column (Bio-Rad No. 732-6200, Hercules, CA) according to the manufacturer's instructions. DTT was added to the eluted probe to a final concentration of 10 mM. The specific activity of each probe was determined by scintillation counting. Aliquots of each labeled probe were stored at −80°C. For use, probes were diluted in hybridization buffer, such that 1×10^6 – 2×10^6 dpm were applied to each brain slice.

Hybridization and detection. *Scn1b*$^{flox/+}$ and *Scn1b*$^{del/del}$ littermate mice were killed at P17-18. Fresh frozen mouse brains were sectioned at 20 μm on a cryostat and mounted on Fisher Superfrost plus slides. Slides were then stored at −80° C. For in situ hybridization, slides were removed from the −80°C freezer, placed directly into 4% paraformadehyde for 1 h at room temperature, and then washed three times in 2× SSC for 5 min each. Slides were then incubated in 0.1 M triethanolamine, pH 8.0, containing 0.25% vol/vol acetic anhydride, for 10 min at room temperature. Slides were washed briefly in water and dehydrated through a graded series of ethanol (50–100%) and allowed to air dry. Each brain slice on the slide was hybridized with 70 μl of ^{35}S-labeled sense or antisense cRNA probe (containing 1×10^6 – 2×10^6 dpms) in 50% formamide hybridization buffer, coverslipped, and incubated overnight in a Saran-wrapped box in a 55°C hybridization oven. The next day, coverslips were removed and slices were washed three times in 2× SSC followed by RNAse A digestion (200 μg/ml) for 1 h at 37°C. Slides were then washed in 2× SSC, 1× SSC, and 0.5× SSC, incubated in the 0.1× SSC for 1 h at 70°C, rinsed with water, dehydrated through a graded series of ethanol (50–100%), and allowed to air dry. Labeled slides were exposed to Kodak film for 2–7 days at room temperature. Selected slides were coated with photographic emulsion (NTB2 Eastman Kodak, Rochester, NY) for 7–14 days at 4°C, developed, and counterstained with cresyl violet.

Western Blot Analysis

Scn1b$^{flox/+}$ and *Scn1b*$^{del/del}$ littermates, as well as *Scn1b* wild-type and null littermate mice (Chen *et al.*,

2004), were killed at P17-18. Brains were immediately removed and membranes were prepared as previously described (Isom *et al.*, 1995). Complete protease inhibitor tablets (Roche, Indianapolis, IN), at twice the recommended concentration, were added to all solutions to prevent sodium channel degradation. Rat brain membranes, that had been previously prepared and stored at −80°C, were used as a positive control. Western blot analysis of equal aliquots of protein was then performed as described previously (Malhotra *et al.*, 2000). A rabbit polyclonal antibody raised against the C-terminus of Scn1b (1:500 dilution) was used to detect Scn1b protein.

ACKNOWLEDGMENT

We thank Ms. Audrey Speelman for technical assistance.

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