genesis 39:234–239 (2004)

TECHNOLOGY REPORT

Floxed Allele for Conditional Inactivation of the Voltage-Gated Sodium Channel *Scn8a* ($Na_V1.6$)

Stephen I. Levin[1,2] **and Miriam H. Meisler**[1]*

[1]Department of Human Genetics, University of Michigan School of Medicine, Ann Arbor, Michigan
[2]Unit for Laboratory Animal Medicine, University of Michigan School of Medicine, Ann Arbor, Michigan

Received 20 February 2004; Accepted 21 April 2004

Summary: The sodium channel gene *Scn8a* encodes the channel $Na_V1.6$, which is widely distributed in the central and peripheral nervous system. $Na_V1.6$ is the major channel at the nodes of Ranvier in myelinated axons. Mutant alleles of mouse *Scn8a* result in neurological disorders including ataxia, tremor, paralysis, and dystonia. We generated a floxed allele of *Scn8a* by inserting loxP sites around the first coding exon. The initial targeted allele containing the neo-cassette was a severe hypomorph. In vivo deletion of the neo-cassette by Flp recombinase produced a floxed allele that generates normal expression of $Na_V1.6$ protein. Ubiquitous deletion of the floxed exon by Cre recombinase in ZP3-Cre transgenic mice produced the *Scn8a*^{del} allele. The null phenotype of *Scn8a*^{del} homozygotes confirms the in vivo inactivation of *Scn8a*. Conditional inactivation of the floxed allele will make it possible to circumvent the lethality that results from complete loss of *Scn8a* in order to investigate the physiologic role of $Na_V1.6$ in subpopulations of neurons. genesis 39:234–239, 2004.

Key words: Scn8a; $Na_V1.6$; conditional allele; Cre/loxP

Voltage-gated sodium channels generate the rapidly rising phase of action potentials in the cell membranes of electrically excitable cells such as neurons and myocytes (Hille, 2001). The mammalian genome contains 10 voltage-gated sodium channel genes which differ in tissue distribution, subcellular localization, and kinetic properties (Meisler *et al.*, 2001; Yu and Catterall, 2003). Voltage-gated sodium channel α subunits are 260-kDa proteins consisting of four homologous domains each with six transmembrane segments. A positively charged transmembrane segment in each domain serves as the voltage sensor. The extracellular loops between segments five and six reenter the membrane and form the ion selectivity filter (Catterall, 2000).

Scn8a, encoding the sodium channel protein $Na_V1.6$, is widely expressed in neurons throughout the central and peripheral nervous system (Burgess *et al.*, 1995; Schaller *et al.*, 1995). $Na_V1.6$ is the primary sodium channel at the nodes of Ranvier in myelinated axons (Caldwell *et al.*, 2000). Additional subcellular sites of $Na_V1.6$ localization include axon initial segments and axons and dendrites of unmyelinated neurons (Caldwell et al., 2000; Jenkins and Bennett, 2001; Black *et al.*, 2002; Boiko et al., 2003). Null mutations of *Scn8a* in the mouse result in ataxia, tremor, and progressive paralysis with lethality by 3 weeks of age (Meisler *et al.*, 2001). Reduced nerve conduction in myelinated axons and failure of evoked neurotransmitter release at neuromuscular junctions of null mice result in "functional denervation" of muscle (Duchen and Stefani, 1971; Angaut-Petit *et al.*, 1982; Kearney *et al.*, 2002). Electrophysiological abnormalities of cerebellar Purkinje cells, pyramidal neurons, motor neurons, the subthalamic nucleus, and the dorsal cochlear nucleus have been observed in mice deficient for $Na_V1.6$ (Raman *et al.*, 1997; Chen *et al.*, 1999; Maurice *et al.*, 2001; Khaliq *et al.*, 2003; Do and Bean, 2004). The findings suggest an important physiologic role for $Na_V1.6$ in spontaneously firing neurons.

The hypomorphic allele *Scn8a*^{medJ} reduces *Scn8a* mRNA and protein to 10% of normal levels (Kearney *et al.*, 2002). *Scn8a*$^{medJ/medJ}$ homozygotes exhibit ataxia and tremor and develop a progressive dystonic phenotype that resembles primary torsional dystonia in humans (Hamann *et al.*, 2003). Because the *Scn8a* deficiency affects many neuronal populations, the physiologic basis of the dystonia is unclear. In an effort to understand the physiologic role of *Scn8a* in the development of neurological disease, and to circumvent the lethality associated with complete loss of this gene, we generated mice carrying the conditional *Scn8a*^{flox} allele.

* Correspondence to: Miriam Meisler, Ph.D., Department of Human Genetics, 4909 Buhl Box 0618, University of Michigan, Ann Arbor, MI 48109-0618.
E-mail: meislerm@umich.edu
Contract grant sponsor: National Institute of Neurological Disorders and Stroke (NINDS), Contract grant number: RO1 NS34509, Contract grant sponsor: Michigan Program in Biomedical Research Training for Veterinary Scientists.
Published online in
Wiley InterScience (www.interscience.wiley.com)
DOI: 10.1002/gene.20050

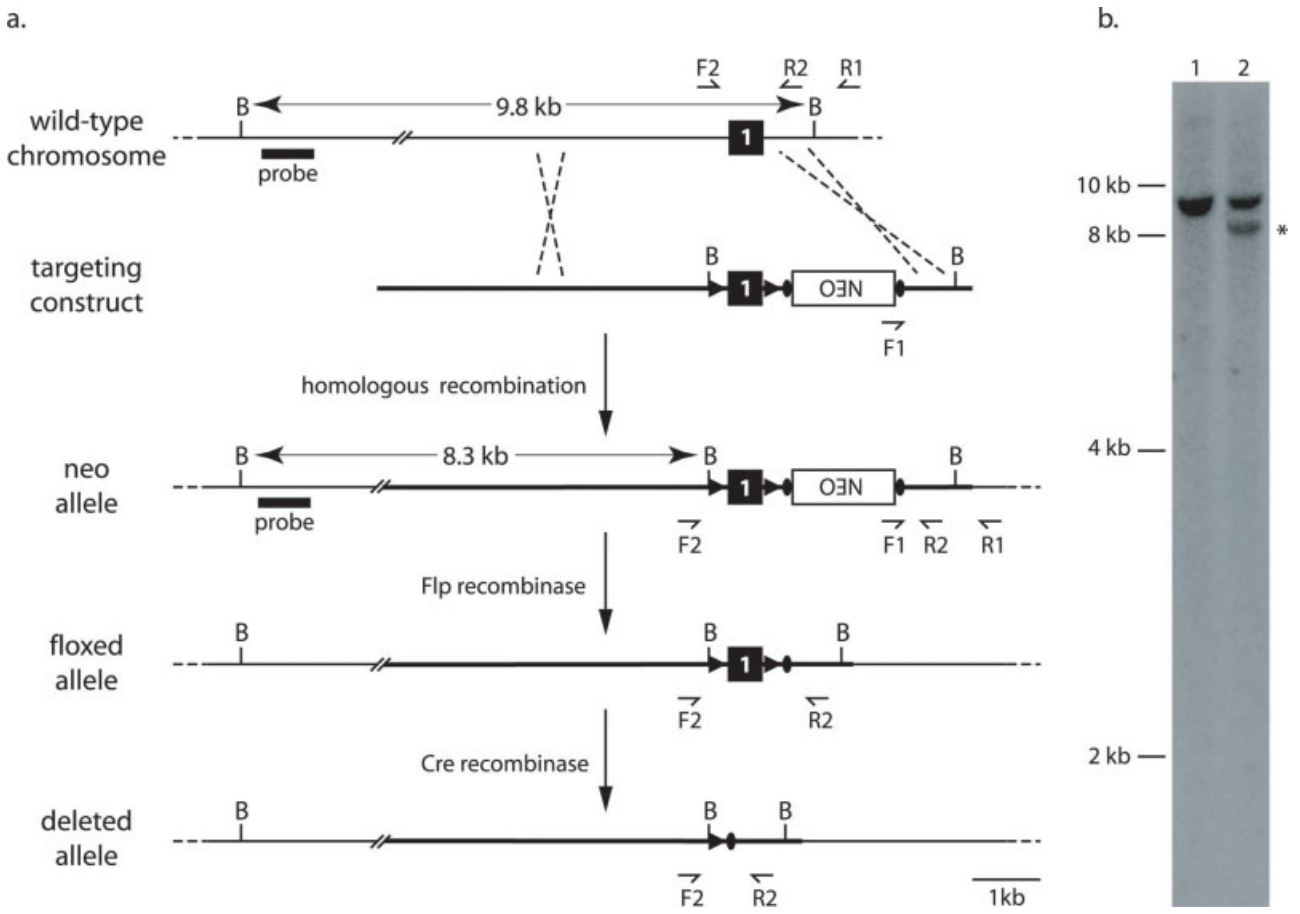

FIG. 1. Targeting of *Scn8a*. **a:** The targeting construct containing exon 1 flanked by loxP sites (triangles) and an inverted PGK-neo-cassette flanked by FRT sites (ovals). There is 5.0 kb of homologous sequence in the left arm and 0.9 kb in the right arm. The Southern probe and diagnostic *Bam*H1 (B) restriction sites are indicated. Correct targeting of the chromosome results in apposition of primer pair F1/R1, which was used for the primary screen of ES cells. Primer pair F2/R2 distinguishes the wildtype, floxed, and deleted alleles but does not amplify the neo allele. **b:** Southern blot of ES cell genomic DNA digested with *Bam*H1. Lane 1, wildtype. Lane 2, correctly targeted. Introduction of a *Bam*H1 site from the targeting vector generates the novel 8.3 kb fragment (*) from the 9.8 kb wildtype fragment.

Scn8a is composed of 28 coding exons (Plummer *et al.*, 1998) spanning ~150 kb of genomic DNA. We introduced two loxP sites to positions 107 bp upstream and 134 bp downstream of exon 1, the first coding exon. Deletion of exon 1 from the related gene *Scn2a* ($Na_V1.2$) was previously shown to produce a null allele (Planells-Cases *et al.*, 2000). The floxed exon and two homology arms of 5.0 kb and 0.9 kb were assembled in a vector containing a neo-cassette flanked by FRT sites (Fig. 1a). The FRT sites were included to permit in vivo deletion of the neo-cassette if its presence interfered with expression of the targeted allele (Dymecki, 1996).

The targeting vector was electroporated into R1 ES cells from mouse strain 129 (Nagy *et al.*, 1993) and 480 neomycin-resistant colonies were recovered. The colonies were screened by PCR using primers F1 and R1 (Fig. 1a). Positive colonies were confirmed by Southern blotting with a probe located upstream of the 5′ homology arm (Fig. 1a,b). Two homologous recombinants were obtained, 4A5 and 5A5, giving a targeting efficiency of 0.4%. Both ES cell lines were injected into B6(D2B6F1) blastocysts to produce chimeric mice. Germline transmission was obtained from 50% of the chimeric founders (9/22). Further analysis was carried out with chimeric mouse 5A5-3, which transmitted the targeted allele to 88% of his offspring.

Crossing mouse 5A5-3 to strain C57BL/6J mice generated $Scn8a^{neo/+}$ mice heterozygous for the neo allele shown in Figure 1. To determine whether the neo-cassette interfered with *Scn8a* expression, these mice were bred to homozygosity. $Scn8a^{neo/neo}$ mice exhibited ataxia, tremors, progressive paralysis, and lethality by 3 weeks of age. This typical *Scn8a* null phenotype suggested that the neo-cassette prevented gene expression, possibly due to the presence of cryptic splice donor and acceptor sites (Jacks *et al.*, 1994). This was confirmed by analysis of RT-PCR products obtained with a forward primer in exon 1 and a reverse primer in exon 3. Het-

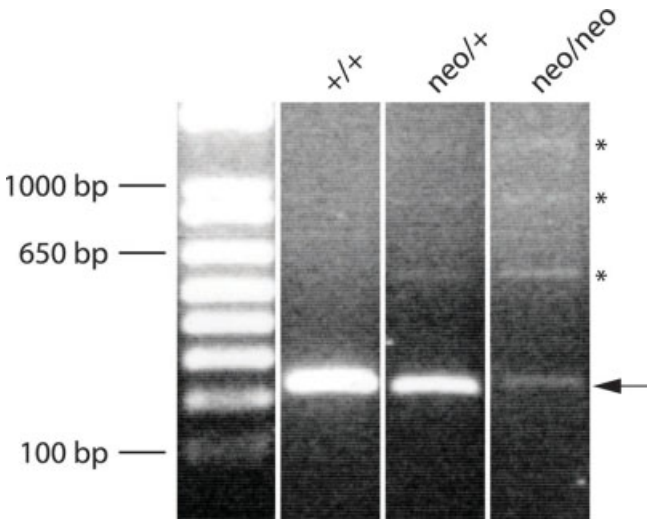

FIG. 2. RT-PCR of brain RNA from *Scn8a*^{neo} mice. Amplification from exon 1 to exon 3. The products obtained from heterozygous *Scn8a*$^{neo/+}$ and homozygous *Scn8a*$^{neo/neo}$ brain reveal a reduction in the correctly spliced product (arrow) and the generation of novel transcripts (*).

erozygous *Scn8a*$^{neo/+}$ mice produce approximately half of the normal amount of correctly spliced transcript, and in homozygotes the transcript is dramatically reduced (Fig. 2). In addition to the correctly spliced product, several longer RT-PCR products were obtained from *Scn8a*$^{neo/+}$ and *Scn8a*$^{neo/neo}$ mice, suggesting that some of the neomycin sequence was incorporated into the transcript. The lethality of *Scn8a*$^{neo/neo}$ mice is consistent with the RT-PCR evidence that this allele is a severe hypomorph, since mice with as little as 10% of correctly spliced transcript are known to be viable (Kearney *et al.*, 2002).

To delete the neo-cassette from the targeted allele, chimeric mice were crossed with mice expressing Flp recombinase (Rodriguez *et al.*, 2000). Offspring were genotyped by amplification of genomic DNA with primers F2 and R2, shown in Figure 1. DNA from *Scn8a*$^{flox/+}$ offspring generated the 800-bp wildtype product and a 900-bp product from the floxed allele (Fig. 3a). The floxed allele retains two lox P sites (Fig. 1).

To evaluate the effect of the remaining loxP sites on expression of the floxed allele, the *Scn8a*$^{flox/+}$ mice were intercrossed to generate *Scn8a*$^{flox/flox}$ homozygotes. *Scn8a*$^{flox/flox}$ mice are viable and did not exhibit any neurological abnormalities. A normal amount of $Na_V1.6$ protein was detected by Western blot of extracts from *Scn8a*$^{flox/flox}$ brains (Fig. 3b).

To determine whether deletion of exon 1 would inactivate *Scn8a*, homozygous *Scn8a*$^{flox/flox}$ mice were crossed with ZP3-Cre mice expressing Cre recombinase in the female germline (Lewandoski *et al.*, 1997). The predicted deleted allele, shown in Figure 1, was detected in heterozygous offspring by amplification of a 300-bp fragment from genomic DNA (Fig. 3a). Intercrosses between heterozygous *Scn8a*$^{del/+}$ mice generated affected mice which exhibited ataxia by P14, followed by paralysis of the hindlimbs (Fig. 4). The affected mice died between 3 and 4 weeks of age. All of the affected mice were homozygous for the deleted allele. The phenotype of the *Scn8a*$^{del/del}$ mice, which is characteristic of other null alleles (Meisler *et al.,* 2001), demonstrates successful Cre-mediated inactivation of *Scn8a*.

The *Scn8a* transcript lacking exon 1 was detected in brain by RT-PCR using a forward primer in the 5′ UTR and a reverse primer in exon 3. A 675-bp product was obtained from wildtype RNA, while a 350-bp product was obtained from *Scn8a*$^{del/del}$ RNA (Fig. 3c). Sequencing the RT-PCR products confirmed the absence of exon 1 from the 350-bp product and demonstrated direct splicing from the noncoding exon (NTE1) to exon 2. Analysis of protein extracts by Western blot did not detect any $Na_V1.6$ protein in brain of *Scn8a*$^{del/del}$ mice (Fig. 3b).

We generated a floxed allele of *Scn8a* that can be inactivated by expression of Cre recombinase. The floxed allele produces normal levels of $Na_V1.6$ protein, and homozygotes for this allele do not exhibit neurological abnormalities. In vivo deletion of exon 1 by Cre recombinase results in complete loss of the channel protein. Mice carrying this *Scn8a*^{flox} allele will be useful for dissecting the physiologic consequences of inactivating *Scn8a* in specific types of neurons. Conditional regulation of *Scn8a* inactivation will make it possible to evaluate the cognitive effects of reduced *Scn8a* activity in the CNS, using tests that require mice older than 3 weeks of age. *Scn8a* is known to be expressed at a low level in glia, but its function in these cells in not known. Crosses with a glia-specific Cre would make it possible to investigate this problem. Finally, we hope to use the floxed allele to dissect the contributions of central and peripheral nervous systems to the movement disorders that are the major abnormality in *Scn8a* null mice.

MATERIALS AND METHODS

Construction of the Targeting Vector

BAC clone 270F06 was isolated from a library of genomic DNA from strain 129/SvJ prepared from ES cell line RW4 (Incyte Genomics, Palo Alto, CA). PCR pools from the library were screened with primers from exon 1 of *Scn8a*. Three genomic fragments were used to assemble the targeting construct: a 5′ flank (5.0 kb), an exon 1 fragment (0.5 kb), and a 3′ flank (0.9 kb). These fragments were amplified by PCR from purified BAC DNA using the Expand High Fidelity PCR system (Roche Molecular Biochemicals, Mannheim, Germany). Each fragment was cloned into pCR-XL-TOPO vectors (Invitrogen, Carlsbad, CA). The fragment containing exon 1 was sequenced to verify that the PCR had not introduced coding or splice site mutations. The genomic fragments were excised from

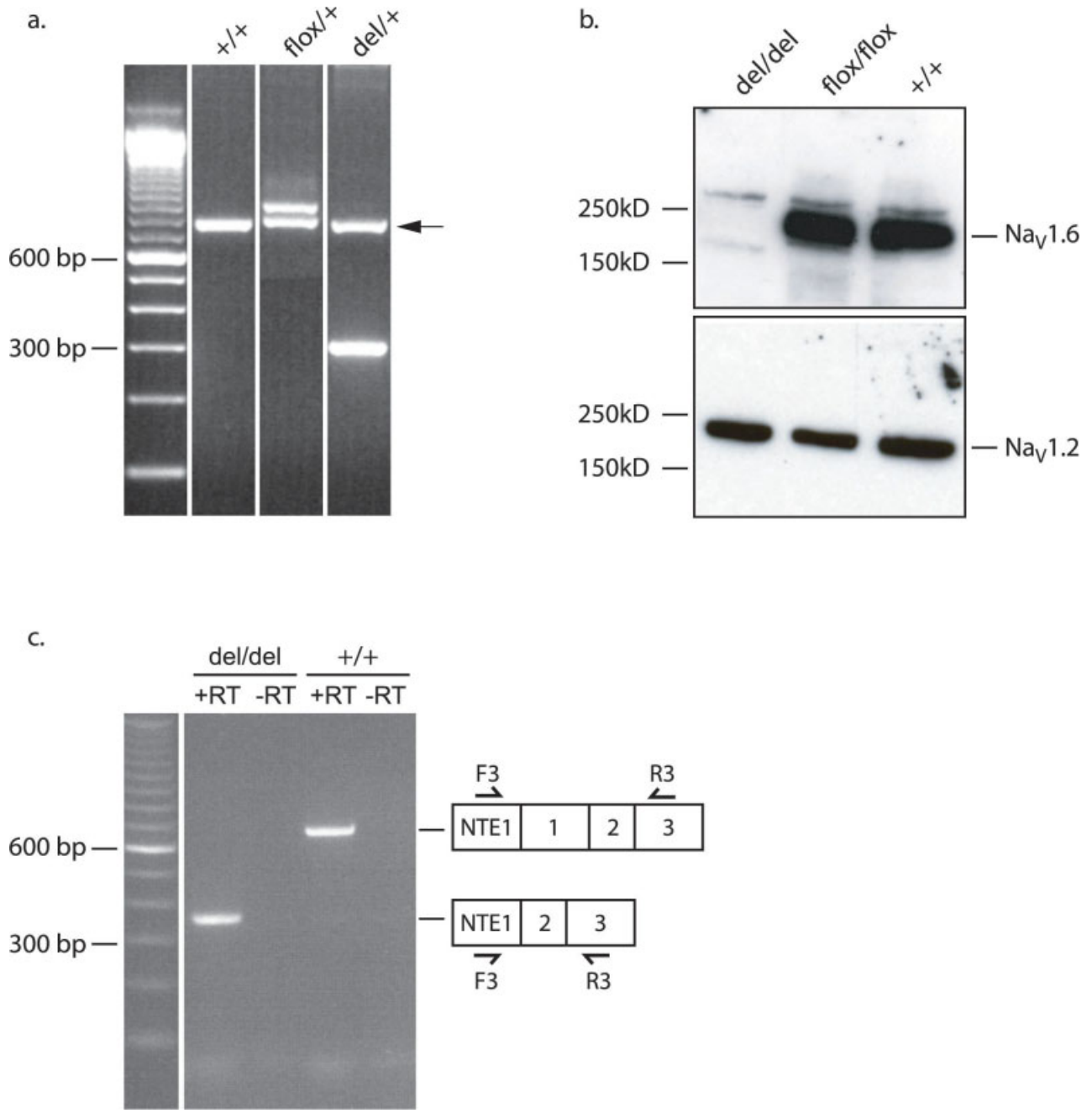

FIG. 3. Characterization of the *Scn8a*^{flox} and *Scn8a*^{del} alleles. **a:** PCR of genomic DNA using primer pair F2/R2. The wildtype allele generates an 800-bp product (arrow), the floxed allele a 900-bp product, and the deleted (del) allele a 300-bp product. **b:** Western blot of brain protein. The blot was probed first with anti-*Scn8a* antibody ($Na_V1.6$), washed, and reprobed with anti-*Scn2a* antibody ($Na_V1.2$) as a control for protein loading. **c:** RT-PCR of *Scn8a* transcripts from brain. Primer locations are indicated. The 5′ UTR of the *Scn8a* transcript is encoded by the exon designated NTE1.

the pCR-XL-TOPO clones, gel purified, and sequentially ligated into the plasmid ploxP-2FRT-PGKneo (kindly provided by David Gordon, University of Colorado Health Sciences Center). A second loxP site was isolated from the plasmid pBS64 (Sauer *et al.*, 1987) and introduced between the 5′ homology arm and the exon fragment.

Screening ES Cells

Genomic DNA from ES cell colonies was screened by PCR using primer pair F1 (5′-GGA ATA GGA ACT TCG TTC TGC TCG-3′) in the neomycin gene and R1 (5′-ATG GGA CCC TGG GGA CCA CAC TTA-3′), located downstream of the 3′ homology arm. Positive clones were

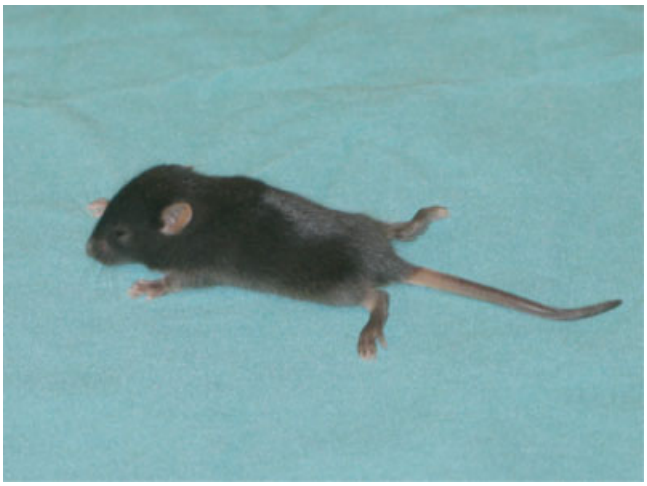

FIG. 4. *Scn8a* null phenotype of *Scn8a*$^{del/del}$ homozygotes. Muscle weakness and hindlimb paralysis is demonstrated by the abnormal posture and splayed hindlimbs of this homozygote at P18. Affected mice do not survive beyond 1 month of age.

analyzed by Southern blot of *Bam*HI digested DNA, using a probe located upstream of the 5′ homology arm (Fig. 1a).

PCR Analyses

Floxed and deleted mice were genotyped using primer pair F2 (5′-GTG TGT GAT TCT CAA CAG TGG GTT-3′) and R2 (5′-GTC TGT AAG AAG GCC TGA AAG TGA-3′) located upstream of exon 1 and within intron 1, respectively. RT-PCR was performed with forward primer F3 in the first nontranslated exon, NTE1, (5′-CAC TGA GGT TTG GAC AGG TTG TCA CC-3′) or F4 in exon 1 (5′-GGA CTT TGA CCC GTA CTA TTT GAC-3′) and reverse primer R3 in exon 3 (5′-ATA CAC AGT TGG TCA GGA TGG TGC-3′).

Western Blot

The membrane protein fraction was isolated from brain homogenates and Western blots were carried out as described previously (Isom *et al.*, 1995). The blot was incubated overnight with rabbit polyclonal anti-*Scn8a* (1:200; Sigma, St. Louis, MO) or rabbit polyclonal anti-*Scn2a* (1:200; Sigma) followed by a 1-h incubation with a peroxidase-conjugated mouse antirabbit antibody (1:200,000; Jackson ImmunoResearch, West Grove, PA). Signal was visualized by chemiluminescent detection with SuperSignal West Femto (Pierce, Rockford, IL).

ACKNOWLEDGMENTS

We thank D. Gordon for providing the ploxP-2FRT-PGK-neo clone, P. Gage for providing the pBS64 clone, E. Hughes and V. Zawistowski for assistance with mouse ES cell culture, and T. Saunders and K. Childs for production of ES cell-mouse chimeras.

LITERATURE CITED

Angaut-Petit D, McArdle JJ, Mallart A, Bournaud R, Pincon-Raymond M, Rieger F. 1982. Electrophysiological and morphological studies of a motor nerve in 'motor endplate disease' of the mouse. Proc R Soc Lond B Biol Sci 215:117–125.

Black JA, Renganathan M, Waxman SG. 2002. Sodium channel Na(v)1.6 is expressed along nonmyelinated axons and it contributes to conduction. Brain Res Mol Brain Res 105:19–28.

Boiko T, Van Wart A, Caldwell JH, Levinson SR, Trimmer JS, Matthews G. 2003. Functional specialization of the axon initial segment by isoform-specific sodium channel targeting. J Neurosci 23:2306–2313.

Burgess DL, Kohrman DC, Galt J, Plummer NW, Jones JM, Spear B, Meisler MH. 1995. Mutation of a new sodium channel gene, Scn8a, in the mouse mutant 'motor endplate disease.' Nat Genet 10:461–465.

Caldwell JH, Schaller KL, Lasher RS, Peles E, Levinson SR. 2000. Sodium channel Na(v)1.6 is localized at nodes of ranvier, dendrites, and synapses. Proc Natl Acad Sci U S A 97:5616–5620.

Catterall WA. 2000. From ionic currents to molecular mechanisms: the structure and function of voltage-gated sodium channels. Neuron 26:13–25.

Chen K, Sprunger LK, Meisler MH, Waller HJ, Godfrey DA. 1999. Reduced spontaneous activity in the dorsal cochlear nucleus of Scn8a mutant mice. Brain Res 847:85–89.

Do MT, Bean BP. 2004. Sodium currents in subthalamic nucleus neurons from Nav1.6-null mice. J Neurophysiol, in press.

Duchen LW, Stefani E. 1971. Electrophysiological studies of neuromuscular transmission in hereditary 'motor end-plate disease' of the mouse. J Physiol 212:535–548.

Dymecki SM. 1996. Flp recombinase promotes site-specific DNA recombination in embryonic stem cells and transgenic mice. Proc Natl Acad Sci U S A 93:6191–6196.

Hamann M, Meisler MH, Richter A. 2003. Motor disturbances in mice with deficiency of the sodium channel gene Scn8a show features of human dystonia. Exp Neurol 184:830–838.

Hille B. 2001. Ion channels of excitable membranes, 3rd ed. Sunderland, MA: Sinauer Associates.

Isom LL, Scheuer T, Brownstein AB, Ragsdale DS, Murphy BJ, Catterall WA. 1995. Functional co-expression of the beta1 and type IIA alpha subunits of sodium channels in a mammalian cell line. J Biol Chem 270:3306–3312.

Jacks T, Shih TS, Schmitt EM, Bronson RT, Bernards A, Weinberg RA. 1994. Tumour predisposition in mice heterozygous for a targeted mutation in Nf1. Nat Genet 7:353–361.

Jenkins SM, Bennett V. 2001. Ankyrin-G coordinates assembly of the spectrin-based membrane skeleton, voltage-gated sodium channels, and L1 CAMs at Purkinje neuron initial segments. J Cell Biol 155:739–746.

Kearney JA, Buchner DA, De Haan G, Adamska M, Levin SI, Furay AR, Albin RL, Jones JM, Montal M, Stevens MJ, Sprunger LK, Meisler MH. 2002. Molecular and pathological effects of a modifier gene on deficiency of the sodium channel Scn8a (Na(v)1.6). Hum Mol Genet 11:2765–2775.

Khaliq ZM, Gouwens NW, Raman IM. 2003. The contribution of resurgent sodium current to high-frequency firing in Purkinje neurons: an experimental and modeling study. J Neurosci 23:4899–4912.

Lewandoski M, Wassarman KM, Martin GR. 1997. Zp3-cre, a transgenic mouse line for the activation or inactivation of loxP-flanked target genes specifically in the female germ line. Curr Biol 7:148–151.

Maurice N, Tkatch T, Meisler M, Sprunger LK, Surmeier DJ. 2001. D1/D5 dopamine receptor activation differentially modulates rapidly inactivating and persistent sodium currents in prefrontal cortex pyramidal neurons. J Neurosci 21:2268–2277.

Meisler MH, Kearney J, Escayg A, MacDonald BT, Sprunger LK. 2001. Sodium channels and neurological disease: insights from Scn8a mutations in the mouse. Neuroscientist 7:136–145.

Nagy A, Rossant J, Nagy R, Abramow-Newerly W, Roder J. 1993.

Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. Proc Natl Acad Sci U S A 90:8424–8428.

Planells-Cases R, Caprini M, Zhang J, Rockenstein EM, Rivera RR, Murre C, Masliah E, Montal M. 2000. Neuronal death and perinatal lethality in voltage-gated sodium channel alpha(II)-deficient mice. Biophys J 78:2878–2891.

Plummer NW, Galt J, Jones JM, Burgess DL, Sprunger LK, Kohrman DC, Meisler MH. 1998. Exon organization, coding sequence, physical mapping, and polymorphic intragenic markers for the human neuronal sodium channel gene SCN8A. Genomics 54: 287–296.

Raman IM, Sprunger LK, Meisler MH, Bean BP. 1997. Altered subthreshold sodium currents and disrupted firing patterns in Purkinje neurons of Scn8a mutant mice. Neuron 19:881–891.

Rodriguez CI, Buchholz F, Galloway J, Sequerra R, Kasper J, Ayala R, Stewart AF, Dymecki SM. 2000. High-efficiency deleter mice show that FLPe is an alternative to Cre-loxP. Nat Genet 25:139–140.

Sauer B, Whealy M, Robbins A, Enquist L. 1987. Site-specific insertion of DNA into a pseudorabies virus vector. Proc Natl Acad Sci U S A 84:9108–9112.

Schaller K, Krzemien D, Yarowsky P, Krueger B, Caldwell J. 1995. A novel, abundant sodium channel expressed in neurons and glia. J Neurosci 15:3231–3242.

Yu FH, Catterall WA. 2003. Overview of the voltage-gated sodium channel family. Genome Biol 4:207.