Modeling Human Epilepsy by TALEN Targeting of Mouse Sodium Channel *Scn8a*

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Summary: To evaluate the efficiency of TALEN technology for introducing mutations into the mouse genome we targeted *Scn8a*, a member of a multigene family with nine closely related paralogs. Our goal was to generate a model of early onset epileptic encephalopathy by introduction of the *Scn8a* missense mutation p.Asn1768Asp. We used a pair of TALENs that were highly active in transfected cells. The targeting template for homologous recombination contained a 4 kb genomic fragment. Microinjection of TALENs with the targeting construct into the pronucleus of 350 fertilized mouse eggs generated 67 live-born potential founders, of which 5 were heterozygous for the pathogenic mutation, a yield of 7% correctly targeted mice. Twenty-four mice carried one or two *Scn8a* indels, including 12 frameshift mutations and the novel amino acid deletion p.Asn1759del. Nine off-site mutations in the paralogs sodium channel genes *Scn5a* and *Scn4a* were identified. The data demonstrate the feasibility and efficiency of targeting members of multigene families using TALENs. The *Scn8a*^tm1768DMm^ mouse model will be useful for investigation of the pathogenesis and therapy of early onset seizure disorders. genesis 52:141–148. © 2013 The Authors genesis Published by Wiley Periodicals, Inc.

Key words: encephalopathy; multigene family; mutagenesis; Nav1.6

INTRODUCTION

Mice carrying human pathogenic mutations are valuable tools for analysis of gene function, disease mechanisms, and new therapies. During the past 20 years, many pathogenic mutations have been introduced into the mouse genome by homologous recombination in embryonic stem cells (Menke, 2013). A recently developed alternative technology employs sequence-specific endonucleases to target specific sites for mutagenesis. Zinc finger nucleases, transcription activator-like (TAL) effector nucleases (TALENs), and RNA guided CRISPR/Cas nucleases generate site-specific breaks in complex genomes that are subsequently mis-repaired, either by nonhomologous end-joining to generate small insertions and deletions or by homologous recombination with a mutated template to introduce specific changes into the genome (Meyer *et al*., 2012; Panda *et al*., 2013; Yang *et al*., 2013). Thus far, these methods have been most often used for *in vitro* mutagenesis of cultured cells, including mouse ES cells. A small number of null alleles (Davies *et al*., 2013; Qiu *et al*., 2013; Sung *et al*., 2013) and missense mutations (Panda *et al*., 2013; Wefers *et al*., 2013) have been introduced into the mouse genome.

We used TALENs to generate a mouse model of the missense mutation p.Asn1768Asp in the neuronal sodium channel Nav1.6 encoded by the gene *Scn8a*. This *de novo* mutation was identified in a child with severe, early onset epileptic encephalopathy by whole genome sequencing (Veeramah *et al*., 2012). More than a dozen *de novo* mutations of *SCN8A* have since been identified by exome sequencing of patients with epileptic encephalopathy or intellectual disability, making

Scn8a a significant new source of neurological disease (Allen *et al*., 2013; Carvill *et al*., 2013; Rauch *et al*., 2012). In addition to *de novo* mutations, a heterozygous null mutation of *SCN8A* cosegregated with cognitive impairment in a human pedigree (Trudeau *et al*., 2006). Heterozygous null mutations in the mouse also result in behavioral abnormalities (McKinney *et al*., 2008).

SCN8A is a member of a highly conserved multigene family encoding nine paralogous sodium channels, 7 expressed in neurons and 2 expressed in muscle (Catterall *et al*., 2008; Meisler *et al*., 2010; Zakon, 2012). *SCN8A* encodes the sodium channel Nav1.6, which is abundant in the central and peripheral nervous systems (O'Brien and Meisler, 2013). Nav1.6 is localized at nodes of Ranvier and at the axon initial segment, where it regulates neuronal firing (Boiko *et al*., 2001; Lorincz and Nusser, 2008; Van Wart *et al*., 2007).

The previously described mouse mutants of *Scn8a* result from partial or complete loss of function mutations (O'Brien and Meisler, 2013). In contrast, the human epilepsy mutation p.Asn1768Asp exhibits a dominant gain-of-function due to impaired channel inactivation (Veeramah *et al*., 2012). A mouse model of p.Asn1768Asp will provide novel information regarding the *in vivo* effects of *Scn8a* hyperactivity and the pathogenesis of epileptic encephalopathy.

RESULTS AND DISCUSSION

Six pairs of TALENs were designed to generate a double-stranded break near the targeted nucleotide c.5302A>G in exon 26 of *Scn8a*, and to maximize mismatches with the paralogous sodium channel genes. Only three of these exhibited nuclease activity in transfected NIH3T3 cells. The TALENs in Figure 1A had the highest activity and were selected for microinjection. The targeted nucleotide is located 25 bp downstream from the predicted endonuclease digestion site in the spacer between the two TALENs (Fig. 1A, arrow). The two closest off-site matches identified by the program TAL Effector Nucleotide Targeter 2.0 Paired Target Finder (Doyle *et al*., 2012) are located in the paralogous genes *Scn4a* and *Scn5a* encoding sodium channels expressed in skeletal and cardiac muscle (Fig. 1C).

The targeting template for homologous recombination was constructed by cloning a 320 bp fragment derived from overlapping synthetic oligonucleotides of 190 and 184 bp. The 190 bp oligonucleotide contained 9 single nucleotide differences from the endogenous *Scn8a* gene, including the nonsynonymous A > G substitution encoding the p.Asn1768Asp mutation and a synonymous change in the spacer region that introduces a HincII site (Fig. 1B). Seven more synonymous changes within the TALEN binding sites were introduced in order to minimize redigestion of targeted alleles. The codon usage for each introduced codon

was ≥9%. Two flanking genomic fragments were added to the construct, a 1.5 kb upstream left arm and a 2 kb downstream right arm. The structure of the targeting construct, with restriction sites, PCR primers and hybridization probes, is shown in Figure 2A.

Two rounds of microinjection into fertilized mouse eggs were carried out using 2.5 ng/µl of circular targeting plasmid with two TALEN mRNAs each at 10 ng/µl (200 eggs) or 20 ng/µl (150 eggs). Sixty-seven potential founders were obtained, 20 from the first microinjection and 47 from the second. Mice carrying the introduced mutations were identified by PCR amplification of a 327 bp fragment containing the targeted site followed by digestion with HincII (Fig. 2B). Ten of the 67 mice were positive with this assay. To distinguish between correct targeting of *Scn8a* and random insertion of the targeting construct, these 10 mice were analyzed by Southern blotting of HincII digested genomic DNA. Hybridization with a probe external to the targeting construct detected a 3.5 kb HincII fragment in 5 correctly targeted genomic DNAs (Fig. 2C). The yield of targeted mice was 5/67 (7%). The targeted allele is designated *Scn8a*[tm1768DMm]. Reprobing the Southern blot with a probe internal to the targeting plasmid identified 8 mice carrying random insertions of the targeting construct, recognized by the 2.2 kb HincII fragment from within the construct (Fig. 2C). Three of the random insertions occurred in mice that also carried a correctly targeted allele. The other 5 mice with random insertions had high copy inserts that interfered with molecular analysis of the endogenous *Scn8a* locus, leaving 62 potential founders for analysis of nontargeted mutations of *Scn8a*.

Sequencing the targeted site from these 62 potential founders identified 13 distinct indels in 24 mice (Fig. 3B). Eighteen mice carried a single mutant allele and 6 mice were compound heterozygotes carrying two indels (Table 1). The *Scn8a* indels produced frameshift mutations, with the exception of a 3 bp deletion that occurred independently in 4 mice and resulted in the amino acid deletion p.Asn1759del (Fig. 3B). The 6 mice that were compound heterozygotes for frameshift mutations exhibited the classic *Scn8a* homozygous null phenotype, with hind limb paralysis, muscle wasting and juvenile lethality. The incorporation into the targeting vector of 4 synonymous SNPs per TALEN binding site was apparently effective in preventing redigestion after homologous recombination, since none of the indels carried the synonymous SNPs. Eight mice appeared to be mosaic for two different indels plus the wild-type allele, as indicated by three overlapping sequences from the amplified PCR product that could not be resolved.

To detect off-site mutations in the most closely related genomic sequences, we amplified the corresponding fragments of *Scn4a* and *Scn5a* and identified 2 indels of *Scn4a* and 7 indels of *Scn5a* (Fig. 1C,D). (By
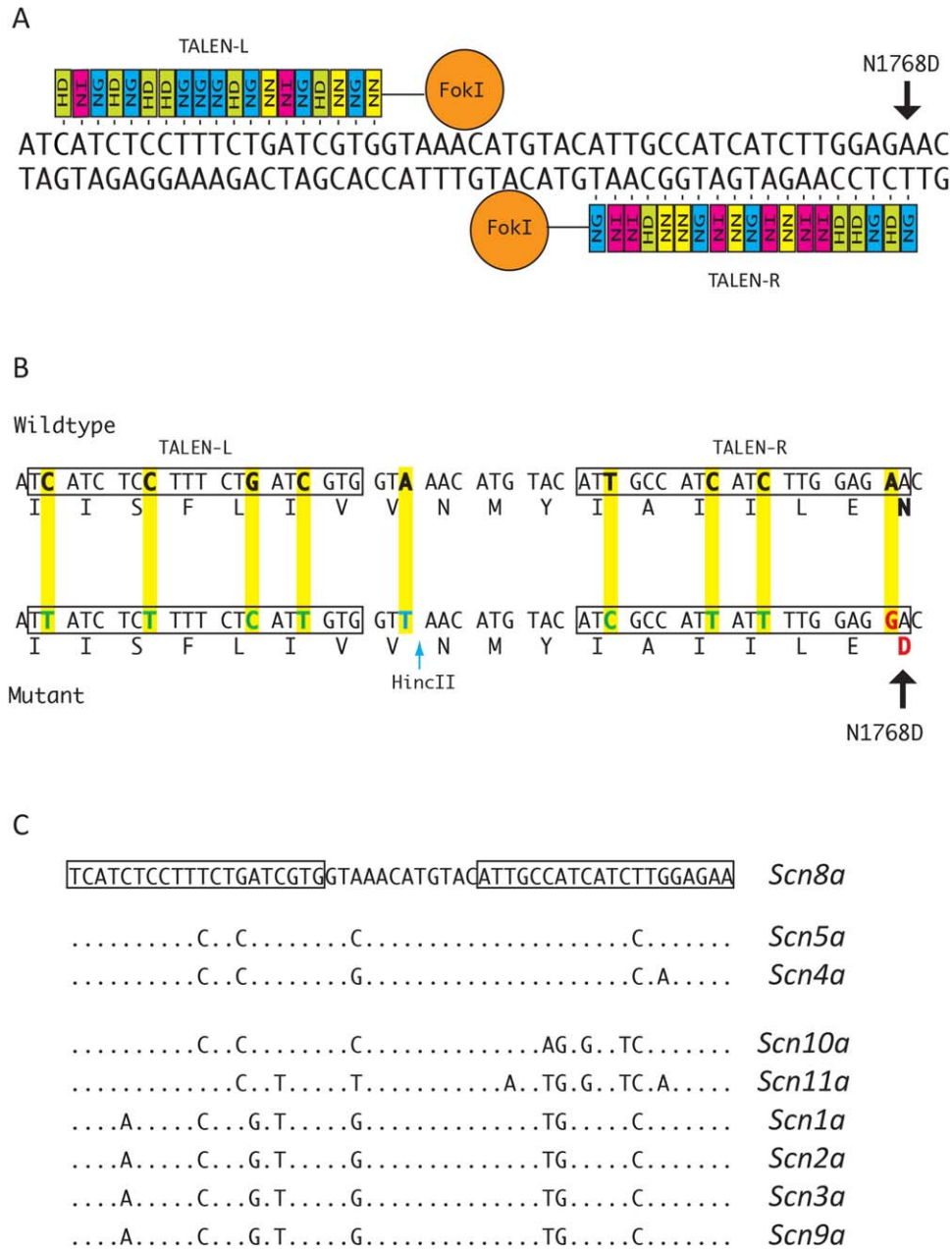
FIG. 1. TALEN binding sites in *Scn8a* and paralogous sodium channel genes. (**A**) Repeat variable diresidue (RVD) sequence for left and right TALENs directing the FokI endonuclease to a site within exon 26 of mouse *Scn8a*. The HD residue (green) binds the C nucleotide; NI (pink) binds A; NG (blue) binds T; NN (yellow) binds G. The targeted nucleotide for introduction of the epilepsy mutation p.Asn1768Asp is marked with an arrow. (**B**) Nine nucleotide substitutions (yellow) were incorporated into the targeting construct to prevent redigestion after homologous recombination with the targeting construct. One substitution introduced the HincII site used for genotyping (blue arrow). (**C**) Sequences of the TALEN targeted site in paralogs from the sodium channel multigene family. Dots represent sequence identity. The most closely related paralogs sites are in *Scn4a* and *Scn5a*, with 1 or 2 bp mismatches per TALEN binding site. The third closest site in the mouse genome is in *Scn10a*, with 5 mismatches in one binding site.

comparison, there were 37 mutant alleles of *Scn8a*). One 9 bp in-frame deletion of *Scn5a* and one 18 bp in-frame deletion of *Scn4a* were observed. The *Scn4a* and *Scn5a* mutations occurred in mice that also carried one or two mutations of *Scn8a* (Table 1), suggesting that a subset of injected eggs expressed a high level of TALEN

activity. No mutations were detected in the more divergent *Scn10a* gene, with 5 mismatches in one TALEN site (Fig. 1).

The highly efficient targeting of *Scn8a* observed in this study confirms the recent evidence that TALEN microinjection is a practical method for introducing
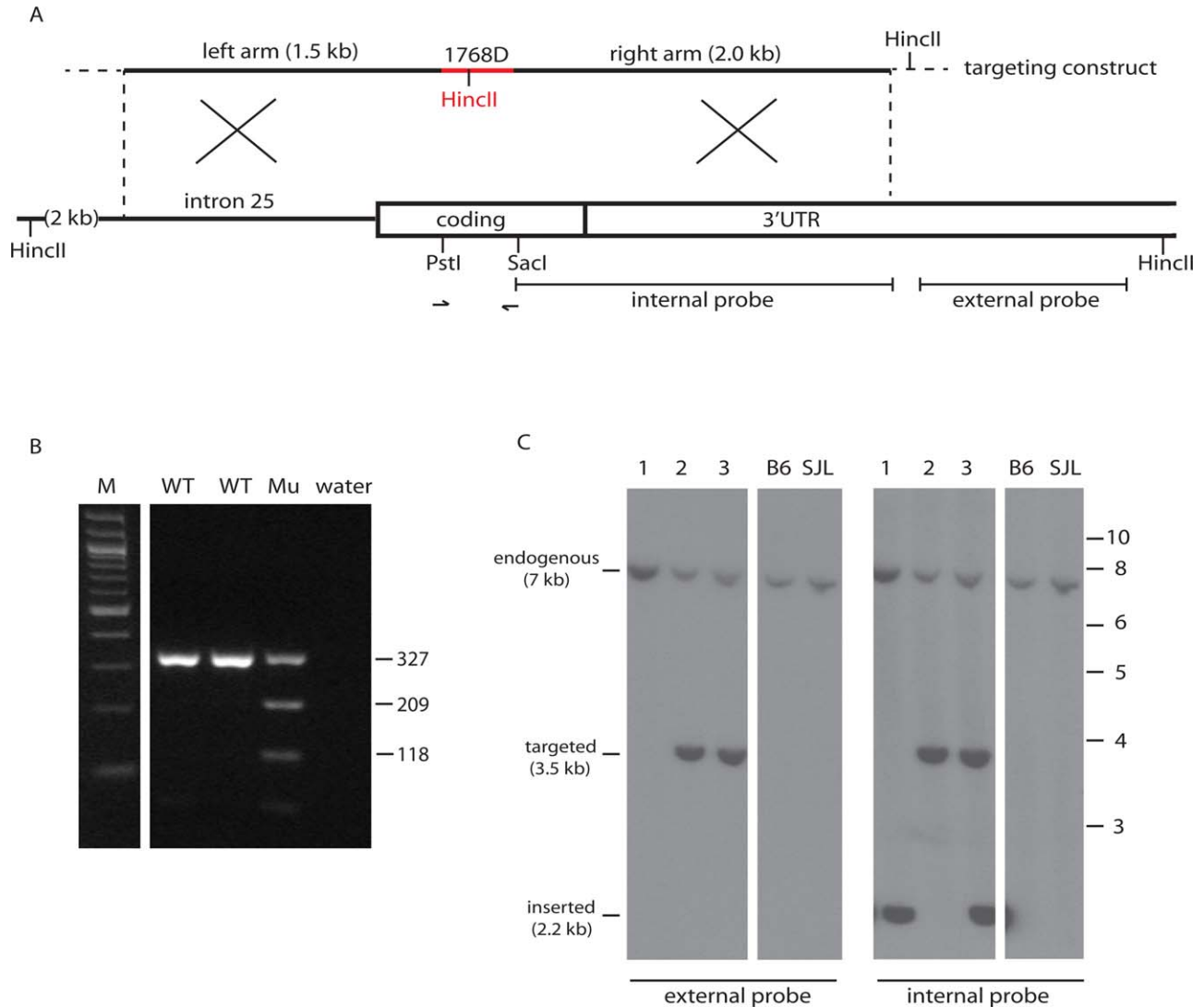
FIG. 2. Structure of the targeting construct and genotype assays to detect targeted alleles and random insertions. (**A**) The 6.3 kb targeting construct was generated from a 320 bp synthetic fragment (red) containing 9 nucleotide substitutions (Fig. 1B) plus flanking genomic fragments of 1.5 and 2.0 kb and the vector sequence of 3 kb (not shown). Arrows represent the PCR primers used to amplify a 327 bp fragment containing the targeted site for genotyping and sequencing. The indicated external probe was used for hybridization of Southern blots with HincII digested genomic DNA, generating a 3.5 kb HincII fragment from correctly targeted *Scn8a* loci. The internal probe was used to detect random insertions of the targeting construct by hybridization to the internal 2.2 kb HincII fragment generated from the HincII site in the vector. (**B**) Detection of mice carrying the HincII site from the targeting vector by PCR amplification of the 327 bp genomic fragment (see A) followed by digestion with HincII. M, 100 bp ladder; WT, wild-type; Mu, mutant with the introduced HincII site. Fragment sizes in bp. (**C**) Distinction between correctly targeted *Scn8a* and random insertion of the targeting construct by Southern blot of HincII digested genomic DNA followed by hybridization with the external probe (left) or the internal probe (right). Three representative genomic DNA samples are shown, containing a targeted allele, a random insertion, or both. MW markers in kb at right.

specific nucleotide substitutions into the mouse genome. The 19% yield of live-born animals (67/350) is comparable to that obtained during routine generation of transgenic mice by microinjection. The 7% yield of mice carrying correctly targeted alleles means that only a single round of microinjection is required to generate a mutation of interest. In contrast, targeting of Scn8a by homologous recombination in ES cells, which do not express *Scn8a*, generated only 2/480 targeted clones

(0.4%) (Levin and Meisler, 2004). The presence of closely related paralogous genes did not produce an excess of off-site mutagenesis, with 37 independent mutations in the *Scn8a* locus and only 9 off-site mutations in the most closely related loci, *Scn4a* and *Scn5a*.

We used a targeting template containing 4 kb of genomic DNA in order to maximize the yield of targeted alleles. Reported yields of 2% correct targeting with a 144 bp oligonucleotide template for a pigmentation

A *Scn8a*[1768D]/+ (n=5)

A T N A T C T C N T T T C T N A T N G T G G T N A A C A T G T A C A T N G C C A T N A T N T T G G A G N A
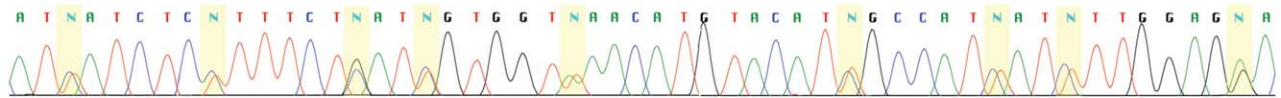
B *Scn8a*[del] (n= 19 + 8 mosaic)

ATC	ATC	TCC	TTT	CTG	ATC	GTG	GTA	AAC	ATG	TAC	ATT	GCC	ATC	ATC	TTG	GAG	AAC	Alleles
I	I	S	F	L	I	V	V	N	M	Y	I	A	I	I	L	E	N	

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ATC ATC TCC TTT CTG ATC GTG GTA A-- ATG TAC ATT GCC ATC ATC TTG GAG AAC   2
ATC ATC TCC TTT CTG ATC GTG GTA AA- -TG TAC ATT GCC ATC ATC TTG GAG AAC   1
ATC ATC TCC TTT CTG ATC GTG GTA AAC --G TAC ATT GCC ATC ATC TTG GAG AAC   2*
ATC ATC TCC TTT CTG ATC GTG GTA AAC A-- TAC ATT GCC ATC ATC TTG GAG AAC   1
ATC ATC TCC TTT CTG ATC GTG GTA --- ATG TAC ATT GCC ATC ATC TTG GAG AAC   4
ATC ATC TCC TTT CTG ATC GTG GT- --- ATG TAC ATT GCC ATC ATC TTG GAG AAC   1
ATC ATC TCC TTT CTG ATC GTG GTA --- -TG TAC ATT GCC ATC ATC TTG GAG AAC   1
ATC ATC TCC TTT CTG ATC GTG GTA --- --G TAC ATT GCC ATC ATC TTG GAG AAC   2
ATC ATC TCC TTT CTG ATC GTG GTA A-- --- TAC ATT GCC ATC ATC TTG GAG AAC   1
ATC ATC TCC TTT CTG ATC GTG --- --- --G TAC ATT GCC ATC ATC TTG GAG AAC   2
ATC ATC TCC TTT CTG ATC GTG GTA AAC ATG TAC ATT GCC ATC ATC TTG GAG AAC   1
                                 ^
                                 A

ATC ATC TCC TTT CTG ATC GTG GTA AAC ATG TAC ATT GCC ATC ATC TTG GAG AAC   1
                                     ^
                                     T

ATC ATC TCC TTT CTG ATC GTG GTA AA. .TG TAC ATT GCC ATC ATC TTG GAG AAC   1
                                 ^
                                 T
```

C *Scn5a*[del] (n=5)

```
ATC ATC TCC TTC CTC ATC GTG GTC --- --- --- ATT GCC ATC ATC CTG GAG AAC   1
ATC ATC TCC TTC CTC ATC GTG GTC A-- ATG TAC ATT GCC ATC ATC CTG GAG AAC   3
ATC ATC TCC TTC CTC ATC GTG GTC AAC --G TAC ATT GCC ATC ATC CTG GAG AAC   1
ATC ATC TCC TTC CTC ATC GTG GTC AAC ATG TAC ATT GCC ATC ATC CTG GAG AAC   1
                                 ^
                                 T

ATC ATC TCC TTC CTC ATC GTG GTC AAC ATG TAC ATT GCC ATC ATC CTG GAG AAC   1
                                 ^
                                 A
```

D *Scn4a*[del] (n=2)

```
ATC ATC TCC TTC CTC ATC GTG GT- --- --- -AC ATT GCC ATC ATC CTA GAG AAC   1
ATC ATC TCC TTC CTC ATC GTG GTC A-- --- --- --- --- --- -TC CTA GAG AAC   1
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FIG. 3. TALEN generated mutations in *Scn8a* and off-site mutations in two sodium channel paralogs. The 327 bp fragment shown in Figure 2A was amplified from the genomic DNA of 62 potential founder mice and sequenced. (**A**) Sequence chromatogram from a founder mouse carrying *Scn8a*[tm1768DMm]. The 9 nucleotide substitutions from the targeting construct in Figure 1B were detected in heterozygous state (yellow). (**B**) Genomic sequence of the *Scn8a* locus from 24 founder mice carrying 13 different indels. (**C, D**) Genomic sequence from 7 founders carrying 5 different indels in the cardiac sodium channel *Scn5a* and two indels in the muscle sodium channel *Scn4a*. The wild-type sequences are shown in Figure 1C. The number of independent alleles observed is indicated at the right. *: homozygote.

Table 1
Genotypes and Allelic Distribution of Sodium Channel Mutations in 62 Offspring Obtained by Microinjection of TALENs and a 4 kb Targeting Construct

Scn8a	Scn5a	Scn4a	# mice
+/+	+/+	+/+	33
tar/+	+/+	+/+	3
tar/−	+/+	+/+	2
+/−	+/+	+/+	15
+/−	+/−	+/+	3
−/−	+/+	+/+	2
−/−	+/−	+/+	2
−/−	+/−	+/−	2
			62

Tar, targeted mutations in the endogenous Scn8a locus; −: null alleles caused by small insertions and deletions; +: wild-type.

gene (Wefers *et al.*, 2013) and 7% correct targeting of *Fus* with a 140 bp oligonucleotide template (Panda *et al.*, 2013), indicate that construction of a targeting vector may be unnecessary for some applications. CRISPR targeting using a targeting construct produced a yield of 10% correctly targeted offspring, but with an apparently higher rate of off-site mutation among the correctly targeted mice (Yang *et al.*, 2013). (We had no off-target mutations in the 5 correctly targeted mice). Thus, for targeting a multi-gene family, TALENs may provide greater specificity.

The p.Asn1768Asp mutation has been transmitted through the germline from two founder mice to 50% of heterozygous offspring (12/25 in one line and 13/22 in the other). The heterozygous p.Asn1768Asp/+ offspring do exhibit the key clinical features of the patient with epileptic encephalopathy, including behavioral abnormalities, seizures, and SUDEP (sudden unexpected death in epilepsy). Unrelated visible phenotypes have not been observed. This new disease model will be valuable for analysis of the neuronal consequences of a hyperexcitable allele of *Scn8a*, and for characterizing its response to antiseizure drugs and other treatments. Detection of novel human mutations by exome sequencing is increasing rapidly. The efficient generation of accurate mouse models using TALEN and related technology will greatly facilitate future investigations of human genetic disorders.

METHODS

Scn8a *Specific TALENs*

Six pairs of *Scn8a*-specific TALENs were designed and tested in transfected NIH 3T3 cells by PNA Bio. (Thousand Oaks, CA). The TALEN constructs employ the standard RVD code and linker between TALE and Fok1 domains (Cermak *et al.*, 2011). Endonuclease activity was evaluated by PCR amplification of *Scn8a* with the primers 8aTAL-F (5′ CCATC TTTGGG ATGTC CAAC 3′) and 8aTAL-R (5′ GGATG TCCAA GCAGT

GGATT 3 ′) as described (http://pnabio.com/products/TALEN.htm). The nucleotide sequences of the two TAL-ENs shown in Figure 1A.

Targeting Construct

The template for homologous recombination was constructed as follows: (1) Synthesis of oligonucleotides of 190 and 184 bp in length with a 34 bp overlap (Integrated DNA technologies). The forward primer contained 9 nucleotide differences from the C57BL/6J genome (Fig. 1B). F oligo (5′ CCCTG ACTGC AGCTT GGACA AGGAG CACCC AGGAA GTGGC TTCAA AGGGG ACTGC GGGAA CCCCT CCGTG GGCAT CTTCT TCTTC GTCAG CTACA TCAT**T** ATCTC **T**TTTC T**CATT** GTGGT **T**AACA TGTAC AT**C**GC CAT**T**A **T**TTTG GAG**G**A CTTCA GCGTA GCCAC AGAGG AAAGC GCCGA TCCTC TGAGC 3′), the R oligo (5′ GGCGA TGAGC TCGAT GGTGT TGGGC TTGGG TACTC GGAGC GGATG CTCCA GGGCG TCGGC AAAGT CGGCC AGCTT ACAGT ACTCG ATGAA CTGGG TGGCA TCAGG ATCAA ACTTC TCCCA GATCT CATAG AAAGT CTCGA GTCG TCCTC GCTCA GAGGA TCGGC GCTTT CCTCT GTGGC TAC 3′). The combined oligonucleotides acted as primer and template in a PCR reaction containing 1× Phusion HF buffer, 0.2 m*M* dNTPs and 1 unit Phusion Taq DNA Polymerase (Thermo Scientific) in 50 μl. The reaction was incubated at 98°C for 30 s followed by 35 cycles of 98°C for 10 s and 72°C for 15 s, with a final 10 min extension at 72°C. The 340 bp product was gel purified, sequentially digested with PstI and SacI to generate a 320 bp fragment, and ligated into the 2.5 kb vector pSP72 (Promega) to generate clone 1. (2) PCR amplification of the 1.5 kb left arm fragment from C57BL/6J genomic DNA was carried out with the forward primer 5′ CACAC ACTCG AGTCT TCTGG CTTAA AACCA TCAGG 3′ and the reverse primer 5′ TCCAA GCTGC AGTCA GGGGG GCGGT TCAGG ATTG 3′. The gel-purified fragment was digested with PstI and XhoI and ligated into clone 1 to generate clone 2. (3) The 2.0 kb right arm fragment was amplified from genomic DNA using the forward primer 5′ CAGTT CATCG AGTAC TGTAA GCTGG 3′ and the reverse primer 5′ACACA CATCG ATCTT ATCTC AGCCA ACACT GCTGT C 3′. The gel purified fragment was digested with SacI and ClaI and ligated into clone 2 to generate the full length 6.3 kb targeting construct.

Microinjection of TALEN mRNA and Targeting Construct into Fertilized Eggs

Polyadenylated and 5′ capped mRNA was synthesized from each TALEN using the mMessage mMachine T7 Ultra Kit and purified using the MEGAclear Kit (Ambion by Life Technologies, Carlsbad, CA). Circular targeting construct DNA (2.5 ng/μl) and TALEN mRNAs (10 or 20 ng/μl each) in 10 m*M* Tris-HCl, 0.1 m*M* EDTA were

microinjected into the pronucleus of (C57BL/6J × SJL)F2 fertilized eggs in the University of Michigan Transgenic Animal Core, Thom Saunders, Director (www.med.umich.edu/tamc/).

Genotyping Potential Founders

Tail biopsies were digested with 1 μg/μl proteinase K in 100 m*M* NaCl, 100 m*M* EDTA, 50 m*M* TrisHCl, pH 8.0, 1% SDS followed by extraction with phenol and chloroform and precipitation with ethanol. A 327 bp genomic fragment containing the targeted site of *Scn8a* was amplified with the primers Tar-F (5′ TGACT GCAGC TTGGA CAAGG AGC 3′) and Tar-R (5′ TCGAT GGTGT TGGGC TTGGG TAC 3′). PCR products were digested with HincII and analyzed on 2% agarose gels to identify mice carrying the introduced HincII site in the TALEN spacer. For Southern blots, 10 μg of DNA was digested with HincII and electrophoresed through 0.8% agarose. Fragments were transferred to Zetaprobe GT nylon membrane (BioRad Laboratories) and hybridized with ^{32}P-dCTP-labeled probes. The external probe for detection of correctly targeted *Scn8a* was amplified from C57BL/6J genomic DNA with the primers 3′UTR-F (5′ CCACT TGGTC TTGTT TCTAC GACG 3′) and 3′UTR-R (5′ CCTAG CAGGA TCAAA TAGCT GTTGC 3′). After autoradiography, the blot was stripped and reprobed with the 2.0 kb right arm DNA fragment (internal probe) to detect random transgene insertions which contain the HincII site from the vector (Fig. 1A). Molecular weight markers, 100 bp ladder and 1 kb ladder, from New England Biolabs.

Sequencing of Targeted Scn8a and Identification of Indels

The 327 bp genomic fragment containing the targeted site of *Scn8a* locus was amplified as described in the preceding section and sequenced from both strands by Sanger Sequencing in the University of Michigan Sequencing Core. The corresponding fragments of *Scn4a* and *Scn5a* were amplified using the primers *Scn4a*-F (5′ CGACA TTGGA GAACC CAGGC AC 3′), *Scn4a*-R (5′ AAGCT TGATC TTATT GGGCT TGGCA 3′), *Scn5a*-F (5′ AACCT GCCCA ACAGC AATGG CTC 3′) and *Scn5a*-R (5′ AACCT GCCCA ACAGC AATGG CTC 3′).

Animals

Mice were housed and cared for in accordance with NIH guidelines. Experiments were approved by the University of Michigan Committee on the Use and Care of Animals. Mutant mice are available to the research community.

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