

GENERAL ARTICLE

Spontaneous seizures and elevated seizure susceptibility in response to somatic mutation of sodium channel *Scn8a* in the mouse

Wenxi Yu¹, Corrine E. Smolen¹, Sophie F. Hill^{1,2} and Miriam H. Meisler^{1,2,*}¹Department of Human Genetics, University of Michigan, Ann Arbor, MI 48109 USA and ²Neuroscience Graduate Program, University of Michigan, Ann Arbor, MI 48109 USA*To whom correspondence should be addressed at: 4808 Med Sci II, 1241 E. Catherine St., Ann Arbor, MI 48109-5618, USA.
Tel: 734 7690124; Email: meislerm@umich.edu

Abstract

De novo mutations of neuronal sodium channels are responsible for ~5% of developmental and epileptic encephalopathies, but the role of somatic mutation of these genes in adult-onset epilepsy is not known. We evaluated the role of post-zygotic somatic mutation by adult activation of a conditional allele of the pathogenic variant *Scn8a*^{R1872W} in the mouse. After activation of CAG-Cre-ER by tamoxifen, the mutant transcript was expressed throughout the brain at a level proportional to tamoxifen dose. The threshold for generation of spontaneous seizures was reached when the proportion of mutant transcript reached 8% of total *Scn8a* transcript, equivalent to expression of the epileptogenic variant in 16% of heterozygous neurons. Expression below this level did not result in spontaneous seizures, but did increase susceptibility to seizure induction by kainate or auditory stimulation. The relatively high threshold for spontaneous seizures indicates that somatic mutation of sodium channels is unlikely to contribute to the elevated incidence of epilepsy in the elderly population. However, somatic mutation could increase susceptibility to other seizure stimuli.

Introduction

Epilepsy is one of the most common neurological disorders and affects between 0.1 and 1% of the population. In approximately one-third of affected individuals, the seizures are refractory to anti-seizure medications (1). Comorbidities may include developmental delay, cognitive disability, sleep disorders and elevated risk of sudden death (2–5). The result is a substantial burden for patients and their families.

Developmental and epileptic encephalopathies (DEEs) are severe disorders characterized by early onset of seizures,

intellectual disability and impaired movement (6). The sodium channel gene *SCN8A* is a significant cause of DEE, with >300 identified *de novo* mutations. Most pathogenic variants of *SCN8A* result in gain-of-function changes in channel activity, including premature channel opening, impaired channel inactivation, elevated persistent current and elevated resurgent current (7–9).

Somatic mutation in post-zygotic cells is increasingly recognized as a source of adult-onset disease (10). Involvement of somatic mutation in focal epilepsy has been directly demonstrated by sequencing surgical samples. Somatic mutation of mechanistic target of rapamycin (mTOR) pathway-related genes

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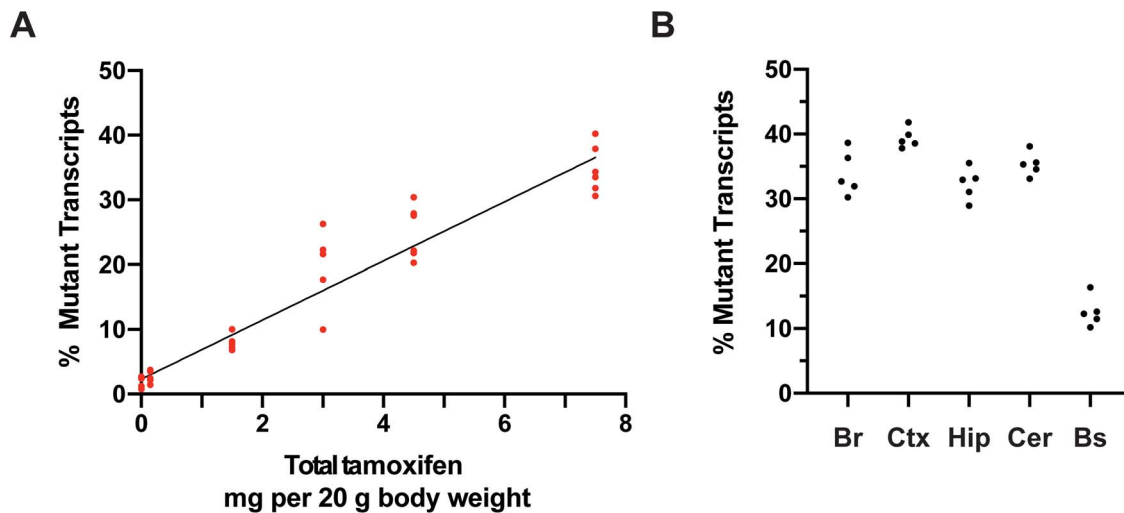


Figure 1. Activation of the tryptophan mutant allele in brain of *Scn8a*^{R1872W} mice. (A) *Scn8a*^{R1872W/+}, *CAG-Cre-ER*^{+/-} mice were treated at 2 months of age with the indicated dose of tamoxifen and evaluated by deep sequencing of an *Scn8a* RT-PCR product containing codon 1872. For read depth and allele frequency, see [Supplementary Material, Table S1](#). (B) Brain distribution of mutant transcripts. Mice were treated with tamoxifen (7.5 mg/20 g body weight). Brain RNA was isolated 1 week after the initiation of tamoxifen treatment. Br, whole brain; Ctx, cortex; Hip, hippocampus; Cer, cerebellum; Bs, brain stem.

is a cause of focal cortical dysplasia and hemimegalencephaly, with epilepsy as a comorbidity (11–14). Somatic mutation of the *SLC35A2* gene encoding a uridine diphosphate-galactose transporter has been identified in seizure foci of epileptic patients (15,16). The number of somatic variants increases with age in prefrontal cortex neurons (17), as does the incidence of epilepsy, which is 2–3x higher in the elderly population than in adults below the age of 60 years (18). The goal of this work was to evaluate the role of somatic mutations of *SCN8A* as a potential cause of adult-onset epilepsy.

p.Arg1872Trp is a recurrent pathogenic mutation responsible for *SCN8A*-DEE (8). *De novo* mutations of arginine 1872 result in delayed channel inactivation and elevated neuronal firing (19,20). We previously generated a conditional knock-in (cKI) mouse model in which *Scn8a*^{R1872W} is activated by Cre recombinase (21). Prenatal activation of *Scn8a*^{R1872W} by *EIIa-Cre* resulted in postnatal seizure onset and death at 2 weeks of age (21). In the current study, we activated expression of *Scn8a*^{R1872W} in adult mice using the tamoxifen-dependent *CAG-Cre-ER*. The data provide insight into the effect of late post-zygotic expression of a known epileptogenic mutation and demonstrate a high threshold for generation of spontaneous seizures in the mammalian brain.

Results

Expression of the conditional *Scn8a*^{R1872W} allele in brain

Mice homozygous for the *Scn8a*-R1872W allele (*Scn8a*^{R1872W/R1872W}) were crossed with *CAG-Cre-ER*^{+/-} mice to generate double heterozygotes with the genotype *Scn8a*^{R1872W/+}, *CAG-Cre-ER*^{+/-}. At 2 months of age, the double heterozygotes were injected intraperitoneally with tamoxifen to generate cKI mice. One week later, brain RNA was isolated. The percent of mutant transcript was quantitated by deep sequencing of a reverse transcription polymerase chain reaction (RT-PCR) product containing the C > T mutation in exon 26 ([Supplementary Material, Table S1](#)). The percent mutant *Scn8a* transcripts in brain were directly proportional to tamoxifen dose in the range of 0.15–7.5 mg/20 g

body weight (Fig. 1A). The maximum theoretical yield of mutant transcript is 50%, as the mice are heterozygous for wild-type and mutant alleles. Treatment with the highest dose resulted in 35% of transcripts containing the mutant sequence (Fig. 1A; [Supplementary Material, Table S1](#)). As the predominant site of expression of *Scn8a* in brain is in neurons (22), we can estimate that 70% of neurons express the mutant transcript in mice receiving the highest dose of tamoxifen.

Seizures began 3 weeks after tamoxifen treatment in mice receiving the highest dose and 10 weeks after tamoxifen in mice receiving 1.5 mg/20 g body weight, the lowest dose that generated seizures (Table 1). To evaluate the distribution of mutant transcript within the brain, RNA was isolated from four dissected brain regions and analyzed by targeted deep sequencing of the RT-PCR amplicon containing the T > C mutation. Mutant transcripts were detected throughout the brain, accounting for 30–40% of transcripts in cortex, hippocampus and cerebellum, and 25% in brain stem (Fig. 1B).

Spontaneous seizures in mice with somatic mutation of *Scn8a*

To determine the effect of activation of *Scn8a*-R1872W, tamoxifen-treated mice were monitored by visual observation from 2 to 8 months of age. The incidence of convulsive seizures was directly correlated with the level of somatic mutation in *Scn8a* (Fig. 2). At the highest doses, with 50–70% of neurons expressing mutant transcripts, all of the mice developed seizures and subsequent death (Fig. 2 and Table 1). At lower doses, with 16–36% of neurons expressing mutant transcripts, only a fraction of the animals developed seizures. At 1 mg/20 g body weight or less, corresponding to ~5% mutant transcripts and 10% affected neurons, no spontaneous seizures were observed (Table 1).

Elevated susceptibility to kainate-induced seizures

To determine whether mice that did not exhibit spontaneous seizures had elevated susceptibility to induced seizures, we examined mice receiving 1 mg/20 g tamoxifen. None of these

Table 1. Seizure phenotypes in *Scn8a* mutant mice

Tamoxifen (mg/20 g body weight)	Mutant transcripts (%)	Neurons with mutant transcripts (%)	Spontaneous seizures	Seizure onset, weeks post-tamoxifen	Lethality at 6 months
0.15	2.6	5.2	0/5	None	0/5
1.0	–	–	0/13	None	0/13
1.5	7.8	16	1/7	5	1/7
3.0	18	36	4/6	10 ± 5	2/6
4.5	25	50	6/6	5 ± 2	5/6
7.5	35	69	14/14	3 ± 1	14/14

Scn8a^{R1872W/+},*CAG-Cre-ER*^{+/-} mice were treated with tamoxifen by intraperitoneal injection. *Scn8a*-R1872W mutant transcripts were determined by targeted deep sequencing 1 week after the first tamoxifen treatment. The maximum possible percent of mutant transcripts in the heterozygous mutant mice is 50%. The proportion of heterozygous neurons expressing the mutant transcript is 2x the percent of mutant transcripts. Mice were monitored for 6 months after tamoxifen treatment.

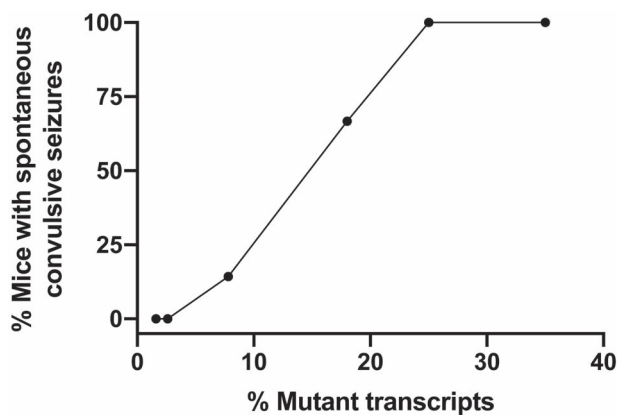


Figure 2. Spontaneous seizures in mice with somatic mutation of *Scn8a*. *Scn8a*^{R1872W/+},*CAG-Cre-ER*^{+/-} mice treated with tamoxifen at 8 weeks of age were monitored for seizures by visual observation for 6 months. The proportion of mutant transcripts was determined by sequencing, as described in Figure 1.

mice developed spontaneous seizures during the 6 months after treatment (0/13; Table 1). Evaluation of susceptibility to seizure induction by kainate was carried out 6 weeks after tamoxifen. Mice received 20 g/kg kainic acid by intraperitoneal injection and were monitored for 2 h. Seizures in the kainate-treated cKI mice were more severe than in littermate controls lacking mutant transcripts (Fig. 3A). Progression to generalized tonic-clonic seizures (GTCSS) was observed in 6/14 cKI mice but only 1/14 control mice ($P = 0.03$, Chi-square test).

Elevated susceptibility to audiogenic seizures

Audiogenic seizures can be induced in cKI mice by brief exposure to a high-frequency auditory stimulus (23). *Scn8a*^{R1872W/+},*CAG-Cre-ER*^{+/-} mice were treated with a low dose of tamoxifen (1 mg/20 g body weight) and exposed to auditory stimulation 6 weeks later. Brief seizure phenotypes were observed in 5/11 of the cKI mice but not in the littermate controls (0/10) (Fig. 3B). Seizure duration was <10 s and no deaths were observed.

Discussion

We describe a model of late post-zygotic mutation of *Scn8a* induced in a conditional mutant by treatment with tamoxifen. When adult *Scn8a*^{R1872W/+},*CAG-Cre-ER*^{+/-} mice were treated at 2 months of age with tamoxifen, the *Scn8a*-R1872W transcript was activated in a dose-dependent manner. At the highest dose of tamoxifen, the mutant transcript was expressed in more than

two-thirds of neurons in cerebellum, cortex and hippocampus. The mutant transcripts are induced in post-mitotic neurons and are not expected to generate a localized focus of mutant neurons. Treated mice develop spontaneous seizures that recapitulate the phenotype of mice with germline mutation of *Scn8a* (21). There was a clear correlation between dose of tamoxifen, percent mutant transcript, time to seizure onset and seizure severity. A threshold of 8% mutant transcripts (equivalent to 16% of neurons expressing the mutant transcript) was required for development of spontaneous seizures which were only seen in a small proportion of mice (1/7). When 50% of neurons expressed the mutant transcript, all of the mice developed seizures (20/20) (Table 1). The requirement for a high proportion of affected neurons is indicative of strong resistance of the normal adult brain to development of seizures. Mice with lower mutation burden did not develop spontaneous seizures, although they did demonstrate increased susceptibility to seizure induction.

Examples from human epilepsy also indicate that higher mutation load results in greater clinical severity. Mosaic parents who transmit a variant allele to an affected child have been identified in ~10% of children with epileptic encephalopathy caused by ion channel mutations (24,25). Among eight parents with mosaicism for *SCN1A*, the variant allele frequency in blood was significantly higher in parents with symptoms compared with unaffected parents (24). In a similar study, one parent with epilepsy had a 29% variant allele frequency in blood samples, compared with <10% in asymptomatic parents (26). Somatic mutation of *SLC35A2*, encoding a UDP-galactose transporter, has been identified in five unrelated patients with intractable seizures (15,16). Only the patients with higher variant allele frequencies exhibited severe intellectual disability and focal cortical dysplasia (15). The variant allele frequency in brain tissue from seizure foci correlated well with electrophysiological seizure severity (16). In patients with hemimegalencephaly, the proportion of mutant alleles varied from 8 to 40% (11).

In the case of inherited germline mutation, 100% of neurons express the mutant transcript. In our experiments, seizures were induced when only 50–70% of neurons express the mutant transcript (Table 1). Mosaicism below 7.8% did not lead to convulsive seizures in any animals, and intermediate levels resulted in partial penetrance of seizures (Table 1). As we only examined convulsive seizures, we cannot rule out the possibility of milder phenotypes such as partial or absence seizures at lower levels of mosaicism. It is also possible that earlier somatic mutation, during childhood, might have a greater impact than the later, adult-induced mutations described here. In the context of gene therapy for inherited germline mutations, our data suggest that amelioration of seizures might require correction >50–65% of neurons.

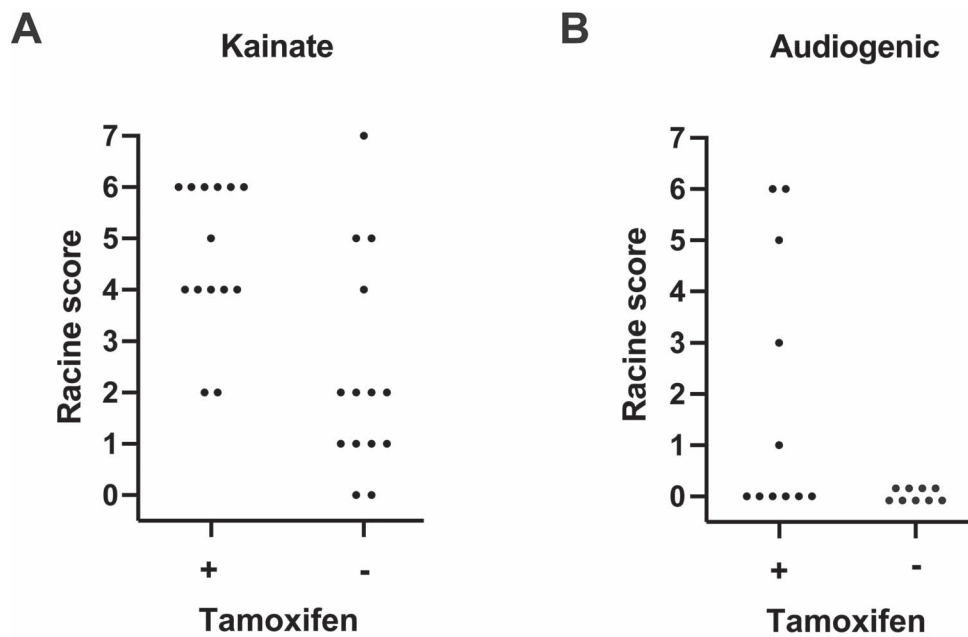


Figure 3. Elevated susceptibility to seizure induction. *Scn8a^{R1872W/+};CAG-Cre-ER^{+/-}* mice were treated with tamoxifen at 1 mg/20 g, which is below the threshold for spontaneous seizures (Table 1). Six weeks later, mice were exposed to the inducing stimulus. Seizure severity was scored using the modified Racine score. (A) Mice were injected with kainic acid (20 mg/kg) and monitored for 2 h. Tamoxifen-treated mice exhibited significantly more generalized seizures (Racine level 6 or 7) than controls (6/14 versus 1/14, $P=0.03$). (B) Mice were exposed to the sound stimulus for 1 min. Brief seizures occurred during the first 15 s of exposure. Tamoxifen-treated mice exhibited more seizure-related events than the controls (5/11 versus 0/9).

There are many differences between this mouse model of tamoxifen-induced somatic mutation and the known features of human somatic mutation. In our model, mutations were induced in individual, isolated post-mitotic cells. In contrast, many pathogenic somatic mutations arise in human neural progenitor cells during development, resulting in clonal populations of mutant daughter cells physically associated in a ‘focus’ with higher potential for altering circuits (27,28). It would be of interest to generate somatic mutations in neural progenitor cells of conditional mice, perhaps by intrauterine treatment with tamoxifen, to evaluate the effect on development of seizures.

In addition, our study, each tamoxifen-induced somatic mutation results in an ‘epileptogenic’ variant (*Scn8a^{R1872W}*). In contrast, spontaneous mutations due to DNA damage and repair error during aging are distributed genome wide over 20000 genes, many of which do not have epileptogenic potential. It is highly unlikely that epileptogenic mutations would arise in a sufficient proportion of neurons (>16%) to cause spontaneous seizures in older adults.

The high threshold for seizure induction by spontaneous mutation indicates that late post-zygotic mutations during human aging are unlikely to be determinants of epilepsy in the elderly. However, the accumulation of somatic mutations in older adults may increase susceptibility to seizure initiation by other factors, such as subthreshold inherited variants or environmental exposure such as traumatic brain injury.

Materials and Methods

Animals

The conditional allele *Scn8a^{R1872W}* contains two copies of the final coding exon of *Scn8a*: a wild-type exon flanked by loxP sites and a downstream copy of the exon that contains the R1872W

variant (21). The action of CRE recombinase deletes the wild-type exon and activates the R1872W allele. The conditional allele was generated by transcription activator-like effector nucleases (TALEN) knock-in to a (C57BL/6 JX SJL/J)F2 zygote (21) and has been maintained for >10 generations of backcrossing to strain C57BL/6 J. C57BL/6 J.CAGCRE-ERTM mice carrying a tamoxifen-inducible Cre transgene were obtained from the Jackson Laboratory (strain #004682). Animal experiments were approved by the University of Michigan and the Unit for Laboratory Animal Medicine at University of Michigan in accordance with the National Institute of Health Guide for the Care and Use of Animals (Protocol # PRO00009797). Principles outlined in the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines and the Basel declaration (<https://www.basel-declaration.org/>) including the 3R concept have been considered when planning the experiments.

Genotyping

The conditional allele *Scn8a^{R1872W}* was genotyped by PCR using forward primer 5′-GCACG TGCTG AAAAA GTGG-3′ and reverse primer 5′-CCTCC TCTTA CCGTG CAGAC-3′, which yield a 414 bp product from the wild-type allele and a 448 bp product from the conditional allele (29). Digestion of the PCR product with KpnI generates fragments of 262 and 186 bp from the conditional allele. *CAG-Cre-ER* was genotyped by PCR amplification of a 215 bp product using forward primer 5′-ACTTA GCCTG GGGGT AACTA AACT-3′ and reverse primer 5′-GGTAT CTCTG ACCAG AGTCA TCCT-3′.

Tamoxifen treatment

A 10 mg/ml solution of tamoxifen in 90% corn oil, 10% ethanol (v/v) was prepared daily. A total of 50 mg of tamoxifen (Sigma

T5648) was suspended in 0.5 ml ethanol plus 4.5 ml of corn oil and incubated at 37°C with occasional vortexing until the solution was clear. Animals received daily injections of 1.5 mg tamoxifen per 20 g body weight in ~200 µl for 1–5 days, until the total desired dose was reached. For doses of 0.15 and 1.0 mg, a stock solution of 1 mg/ml was prepared.

Scoring of seizures

Mice were monitored visually to detect severe GTCSs and seizure-related death. Mice were observed for 7 h per day (9 a.m.–5 p.m.), 7 days per week for 6 months after the first tamoxifen treatment. Seizures were scored according to the modified Racine score (30): 0, no response; 1, freezing; 2, head nodding; 3, Straub tail; 4, forelimb clonus; 5, rearing and falling; 6, GTCS; 7, death.

Targeted deep sequencing of the *Scn8a* transcript

Total RNA was prepared from brain by TRIzol™ extraction using a Direct-zol RNA Miniprep Plus kit (Zymogen R2071). Complementary DNA (cDNA) was synthesized with the LunaScript® RT SuperMix Kit (NEB). A 1011 bp fragment of the *Scn8a* transcript containing the R1872W mutation was amplified from cDNA using forward primer 5'-GGTCA TTCTC TCCAT TGTGG-3' and reverse primer 5'-CCTCC ATTCT CCAGC TTGTT-3'. Targeted deep sequencing of the RT-PCR product was carried out with the Illumina MiSeq system. Raw data was aligned to the mm10 reference genome using HISAT2 (31) and analyzed using SAMtools mpileup (32). The number of sequence reads varied from 700 to 6400 per sample.

Kainic acid treatment

Kainic acid (Cayman Chemical) was dissolved in phosphate-buffered saline at 2.5 mg/ml. Adult *Scn8a*^{R1872W/+}, *CAG-Cre-ER*^{+/-} mice were first treated with a tamoxifen dose of 1.0 mg/20 g body weight to activate the conditional allele. Six weeks after tamoxifen treatment, *Scn8a*^{R1872W/+}, *CAG-Cre-ER*^{+/-} mice and littermate controls lacking Cre were randomly selected and injected intraperitoneally with 20 mg/kg kainic acid. Mice were monitored for 2 h. Seizure severity was scored by an experimenter blinded to genotype. As no sex differences were observed, data from both sexes were combined.

Audiogenic seizures

Two-month-old mice were treated with tamoxifen at 1.0 mg/20 g body weight. Six weeks later, audiogenic seizures were induced by sound generated by a cell sonicator (33,34). Mice were exposed for 1 min to Branson Sonifier Model 185 operated at 50% maximum power. Seizure phenotypes were observed within 15 s after the initiation of exposure. Seizure severity was scored by an experimenter blinded to genotype. Statistical analysis was performed using Student's t-test.

Supplementary Material

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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