Aberrant regulation of a poison exon caused by a non-coding variant in Scn1a-associated epileptic encephalopathy

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Abstract

Dravet syndrome (DS) is a developmental and epileptic encephalopathy that results from mutations in the Na1.1 sodium channel encoded by SCN1A. Most known DS-causing mutations are in coding regions of SCN1A, but we recently identified several disease-associated SCN1A mutations in intron 20 that are within or near to a cryptic and evolutionarily conserved “poison” exon, 20N, whose inclusion leads to transcript degradation. However, it is not clear how these intron 20 variants alter SCN1A transcript processing or DS pathophysiology in an organismal context, nor is it clear how exon 20N is regulated in a tissue-specific and developmental context. We address those questions here by generating an animal model of our index case, NM_006920.4(SCN1A):c.3969+2451G>C, using gene editing to create the orthologous mutation in laboratory mice. Scn1a heterozygous knock-in (+/KI) mice exhibited an ~50% reduction in brain Scn1a mRNA and Na1.1 protein levels, together with characteristics observed in other DS mouse models, including premature mortality, seizures, and hyperactivity. In brain tissue from adult Scn1a +/- animals, quantitative RT-PCR assays indicated that ~1% of Scn1a mRNA included exon 20N, while brain tissue from Scn1a +/KI mice exhibited an ~5-fold increase in the extent of exon 20N inclusion. We investigated the extent of exon 20N inclusion in brain during normal fetal development in RNA-seq data and discovered that levels of inclusion were ~70% at E14.5, declining progressively to ~10% postnatally. A similar pattern exists for the homologous sodium channel Na1.6, encoded by Scn8a. For both genes, there is an inverse relationship between the level of functional transcript and the extent of poison exon inclusion. Taken together, our findings suggest that poison exon usage by Scn1a and Scn8a is a strategy to regulate channel expression during normal brain development, and that mutations recapitulating a fetal-like pattern of splicing cause reduced channel expression and epileptic encephalopathy.

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Author Summary

Dravet syndrome (DS) is a neurological disorder affecting approximately 1:15,700 Americans[1]. While most patients have a mutation in the SCN1A gene encoding Na1.1 sodium channels, about 20% do not have a mutation identified by exome sequencing. Recently, we identified variants in intron 20N, a noncoding region of SCN1A, in some DS patients [2]. We predicted that these variants alter SCN1A transcript processing, decrease Na1.1 function, and lead to DS pathophysiology via inclusion of exon 20N, a “poison” exon that leads to a premature stop codon. In this study, we generated a knock-in mouse model, Scn1a+/KI, of one of these variants, NM_006920.4(SCN1A):c.3969+2451G>C, which resides in a genomic region that is extremely conserved across vertebrate species. We found that Scn1a+/KI mice have reduced levels of Scn1a transcript and Na1.1 protein and develop DS-related phenotypes. Consistent with the poison exon hypothesis, transcripts from brains of Scn1a+/KI mice showed elevated rates of Scn1a exon 20N inclusion. Since Scn1a expression in the brain is regulated developmentally, we next explored the developmental relationship between exon 20N inclusion and Scn1a expression. During normal embryogenesis, when Scn1a expression was low, exon 20N inclusion was high; postnatally, as Scn1a expression increased, there was a corresponding decrease in exon 20N usage. Expression of another voltage-gated sodium channel transcript, Scn8a (Na1.6), was similarly regulated, with inclusion of a poison exon termed as 18N early in development when Scn8a expression was low, followed by a postnatal decrease in exon 18N inclusion and corresponding increase in Scn8a expression. Together, these data demonstrate that poison exon inclusion is a conserved mechanism to control sodium channel expression in the brain, and that an intronic mutation that disrupts the normal developmental regulation of poison exon inclusion leads to reduced Na1.1 and DS pathophysiology.

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Scn1a poison exon inclusion in a mouse model of Dravet syndrome
Introduction

Dravet syndrome (DS) is a developmental and epileptic encephalopathy (DEE) characterized by intractable seizures, developmental delay, speech impairment, ataxia, hypotonia, sleep disturbances, and other health problems [3]. In the U.S., DS incidence is 1 per 15,700 [1], and 73% of patients die before the age of 10 years [4].

The most frequent cause of DS are loss-of-function mutations of SCN1A, which encodes the type I voltage-gated sodium channel (Na\(_{\text{1.1}}\)) alpha subunit, part of a larger family of nine sodium channel proteins (Na\(_{\text{1.1}}\) – Na\(_{\text{1.9}}\)) that control neuronal excitability [5]. Pathogenic SCN1A mutations are generally heterozygous and often occur de novo in DS. DS-associated SCN1A mutations lead to a loss of Na\(_{\text{1.1}}\), which is predominantly expressed in inhibitory GABAergic interneurons, so loss of function leads to network disinhibition [6]. Importantly, the molecular mechanisms for Na\(_{\text{1.1}}\) loss of function differ between various SCN1A mutations; many cause nonsense-mediated RNA decay, while other missense mutations affect Na\(_{\text{1.1}}\) stability or function [5].

Only 80% of DS patients have pathogenic SCN1A variants detectable within coding exons [7], suggesting that variants in noncoding regions near SCN1A may contribute to disease in some patients. A genomic analysis of 640 DEE patients found that five patients harbored rare variants predicted to be deleterious within a highly conserved region deep within SCN1A intron 20 [2]. A 64-bp segment within this region can be alternatively spliced and included as an exon termed 20N [2]. Exon 20N is known as a poison exon because it leads to a truncated and presumably nonfunctional SCN1A isoform due to a stop codon that arises with the frameshift caused by the 64-bp inclusion [8]. Several of the intron 20 variants identified in DEE patients increased inclusion of poison exon 20N in splice reporter assays in non-neuronal cells [2]. As a result, variant-induced aberrant inclusion of SCN1A poison exon 20N has been proposed as a mechanism for Na\(_{\text{1.1}}\) loss of function in DEE patients [2].

Our prior work on non-coding variation and poison exon inclusion in DS was carried out in non-neuronal cultured cells with artificial constructs and did not determine if any of the non-coding variants could recapitulate the phenotype of DS in an organismal context. Here, we report the construction and analysis of a mouse model for a SCN1A variant, NM_006920.4(SCN1A):c.3969+2451G>C (hereafter, c.3969+2451G>C), that we identified in our index patient and that lies within the alternatively spliced poison exon, 20N. [2]. Our results provide rigorous evidence of causality for a non-coding variant, allow direct measurement of poison exon usage in vivo, and give new insight into the normal function of poison exons for sodium channel genes and the consequent relationship to human genetic disease.

Figure 1. The non-coding Dravet Syndrome–causing variant, NM_006920.4(SCN1A):c.3969+2451G>C, is present in a highly conserved region. (A) The alternate exon 20N (shaded rectangle) is highly conserved, with GERP scores that are comparable to canonical exons in SCN1A. (B) Multiple alignment in the 64bp SCN1A 20N region of human, mouse, opossum, alligator, and duck, modified from the Multiz Alignment of 100 Vertebrates track from the UCSC Genome Browser (SFig.1). The red box indicates the position of the variant NM_006920.4(SCN1A):c.3969+2451G>C in our index patient. The red line indicates the position of the guide RNA used for CRISPR/Cas9 gene editing. (C) Alternative splicing of intron 20 in SCN1A. Inclusion of exon 20N (bottom) results in a frame shift and hence a premature termination codon (PTC) in exon 21. The NM_006920.4(SCN1A):c.3969+2451G>C also results in a Gly-Ala (red) substitution within exon 20N. (D) Exon 20N would be in the intracellular loop connecting the fourth and fifth transmembrane voltage sensing regions of the third SCN1A homologous domain (D3) but brings a premature termination codon (PTC) in frame resulting in nonsense-mediated RNA decay.
Results

Evolutionary conservation in intronic regions harboring a DS-causing variant. We first examined evolutionary conservation in the region surrounding the SCN1A intron 20 pathogenic variant that we studied here, c.3969+2451G>C, as a prerequisite to identifying the orthologous variant in mice. Human intron 20 is ~8 kb, within which there exist three highly conserved segments of several hundred nucleotides in length (Fig. 1A). Exon 20N and the surrounding region is highly conserved as indicated by quantitative assessment with genomic evolutionary rate profiling (GERP) (Fig. 1B) and alignment across 77 vertebrates (S1 Fig.). The G>C substitution in our inpatient lies within exon 20N and is perfectly conserved along with neighboring nucleotides in the mouse. SCN1A transcripts that contain this exon 20N are “poisoned” due to a frameshift and consequent premature termination codon in exon 21 (Fig. 1C-D); the same is true for mouse Scn1a.

Scn1a mRNA and protein levels are reduced in the brains of Scn1a+/KI mice. We used CRISPR/Cas9 gene editing [9] to generate mice harboring the c.3969+2451G>C variant on a C57BL/6J background. A guide RNA located upstream of the variant position (Fig. 1B) was used to produce a G>C single nucleotide substitution at the corresponding position in mouse Scn1a, NC_000068.7:g.66293870C>G (GRCh38.p6) (Fig. 2A). All genotypes were confirmed by Sanger sequencing. Animals carrying one allele of the edited variant are termed Scn1a+/KI and compared to non-mutant Scn1a+/+ littermates.

In cortical tissue from postnatal Scn1a+/KI mice, qRT-PCR for an amplicon between exons 19 and 20 (Materials and Methods) indicated an ~50% reduction in levels of Scn1a mRNA (Fig. 2B). Analysis of RNA-seq data from +/- and +/KI animals yielded a similar result (Fig. 2C). We assessed Na1.1 protein levels with antisera targeting C-terminal or N-terminal epitopes; in both cases, levels of full-length protein (260 kDa) was reduced by ~50% (Fig. 2D-I), and there was no evidence of a truncated protein (157 kDa) that would otherwise correspond to the protein predicted from a transcript that contains exon 20N (S2 Fig. A). Taken together, these results indicate that the variant we introduced into Scn1a leads to the absence of Na 1.1, likely due to nonsense-mediated decay of a transcript that contains exon 20N and a downstream premature termination codon.

Scn1a+/KI mice exhibit Dravet syndrome–like phenotypes. A number of SCN1A mutant mouse models have been described previously as models for DS [10-15]. As in patients with DS [16] and prior reports in other DS mouse models [10, 11, 17-21], Scn1a+/KI mice exhibited increased premature mortality compared to Scn1a+/+ littermates, with about 40% mortality at 3 months and 55% mortality between birth and 18 months (Fig. 3A). These figures include only deaths after weaning at P21, when genotypes were obtained. We also noted high mortality rates in these litters prior to weaning and genotyping, so overall premature mortality rates

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are likely even higher than these estimates. We frequently observed spontaneous seizures in Scn1a +/KI mice (video in Supplementary File 1) and the mice did not show signs of declining health, so the premature mortality is likely caused by seizure events incompatible with survival. Further, we never observed homozygous Scn1a KI/KI mice from Scn1a +/KI to Scn1a +/KI intercrosses, suggesting early lethality for homozygosity of this allele, again consistent with other DS mouse models.

We tested Scn1a +/KI mice that survived to adulthood in a battery of behavior assays [22-25] to investigate if they developed behavioral deficits reported in other DS mouse models [26, 27]. Consistent with phenotypes of other DS models [22, 28], Scn1a +/KI mice exhibited hyperactivity in the open field (Fig. 3B-F), including increased distance travelled (Fig. 3B-C) and jumps (Fig. 3D). Notably, percent time in the center of the open field and stereotypic counts were similar in Scn1a +/KI mice compared to Scn1a +/- mice (Fig. 3E-F), indicating no apparent evidence of increased anxiety. In addition, testing in an elevated plus maze, another behavioral assay for anxiety-related phenotypes, [24, 25, 29], revealed no differences in the time spent in open or closed arms, nor total entries into the open and closed arms of the maze (S3A-C Fig.). In a Y-maze assay [24, 25, 30], no differences in spontaneous alternations were observed between Scn1a +/- and +/KI mice (S3D-E Fig.), indicating no apparent deficits in short-term memory. Lastly, Scn1a +/-KI mice did not show any behavioral deficits in the tube test of social dominance [31] (S4A Fig.) or three-chamber sociability test [32] (S4B-D Fig.). Overall, our results on DS-associated behavioral phenotypes in Scn1a +/-KI are similar to what has been reported previously in other DS mouse models. Taken together with the molecular characterization (Fig. 2), these results demonstrate that the mouse models the molecular pathophysiology of a conserved non-coding mutation in exon 20N and provides compelling evidence of its pathogenicity in DS.

Retention of exon 20N in Scn1a +/KI mice. We designed several qPCR primer sets to detect mRNA transcripts either containing or excluding exon 20N (Fig. 4A), after reverse transcription with random primers (Materials and Methods). Amplicon 1 spans from exon 20 to exon 21 and generates a 56-bp product without exon 20N or a 120-bp product when exon 20N is included. In RNA from cortex of animals aged 1.9 mo – 19 mo, levels of the larger transcript reflecting exon 20N inclusion were undetectable in Scn1a +/- mice, but easily detectable in Scn1a +/KI mice (Fig. 4B-C). A second set of primers spans the introns between exons 20 and 20N, and between exons 20N and 21, allowing measurement of exon 20N-containing transcripts as a 96 bp product, ampiclon 2, that can be directly compared to a 111 bp product, ampiclon 3, that spans exons 19 and 20 (Fig. 4A). Expressed as a percentage of ampiclon 2/ampiclon 3, exon 20N is included in 0.97% of Scn1a transcripts in +/- mice, and in 4.8% of Scn1a transcripts in +/KI mice (Fig. 4D). Similar results were obtained after reverse transcription with oligoT. Assuming an additive model in which the presence of the KI allele does not influence activity of the + allele, and vice versa, we conclude that gene-edited variant leads to a ~9 to 10-fold increase in Scn1a transcripts that contain exon 20N. We also note that the levels of normal Scn1a mRNA and protein are reduced ~50% in +/KI compared to +/- mice (Fig. 2), which implies that nearly all
potential function of poison exons in Scn1a and Scn8a.

Aberrant regulation of poison exons as a pathogenetic mechanism, and conservation of that mechanism in humans and mice, raises the more general question of how and why Scn1a poison exons are used normally during development and differentiation. Voltage-gated sodium channels (Na\(_{1.1}\)–Na\(_{1.9}\)) have distinct developmental and regional patterns of expression [33–36]. Na\(_{1.1}\), the alpha subunit encoded by Scn1a, rises after a lag phase to adult levels during the second to fourth postnatal weeks in both mouse and rat brains, and is expressed primarily in a subset of GABAAergic interneurons [33-35]. The homologous protein Na\(_{1.6}\), encoded by Scn8a, has a similar pattern of developmental expression to Na\(_{1.1}\), but is expressed primarily in glutamatergic neurons. Previous work from Meisler and colleagues on Scn8a has identified a poison exon, 18N, whose expression is highest in fetal brain [37]. Inclusion of exon 18N in Scn8a is regulated by several RNA binding proteins [38, 39], and for which a “fail-safe” mechanism has been proposed to prevent the synthesis of active protein in cells or tissues where it would be deleterious. We explored that idea for Scn1a by first measuring expression of alternative isoforms in different tissues of +/+ and +/KI mice using isoform-specific amplicons as shown in Fig. 4A. In heart, kidney, liver, and lung of +/+ and +/KI mice, Scn1a was expressed at very low levels as detected by qRT-PCR (Table 1). We did not have access to fetal tissues from Scn1a +/KI mice, but we analyzed usage of Scn1a exon 20N in non-mutant mice by analyzing a previously generated RNA-Seq dataset of mouse cortex at multiple developmental timepoints [40].

**Table 1. Scn1a expression in various tissues.** Quantification of total Scn1a mRNA levels using amplicon “1” (Figure 3) in Scn1a +/+ and Scn1a +/KI mice (n=1). Low levels of Scn1a mRNA were expressed in lung, liver, kidney, and heart as evidenced by the high \(\Delta\)Ct values. Tbp was used to normalize Scn1a expression. UD = undetectable. Transcripts containing 20N (Amplicon “2” in Figure 3) were undetectable or barely detectable in tissues other than brain.

| Tissue | Genotype   | Scn1a Ct | Tbp Ct | \(\Delta\)Ct | 20N Ct |
|--------|------------|----------|--------|-------------|--------|
| Lung   | Scn1a +/+  | 35.6     | 22.7   | 12.9        | UD     |
|        | Scn1a +/KI | 33       | 22.8   | 10.2        | 36.8   |
| Liver  | Scn1a +/+  | 36.9     | 23     | 13.9        | UD     |
|        | Scn1a +/KI | UD       | 22.4   | -           | UD     |
| Kidney | Scn1a +/+  | 30.5     | 21.5   | 9           | 35.8   |
|        | Scn1a +/KI | 31.1     | 21.8   | 9.3         | 35.2   |
| Heart  | Scn1a +/+  | 36.1     | 25.4   | 10.7        | UD     |
|        | Scn1a +/KI | 34.6     | 24.9   | 9.7         | 38     |
| Brain  | Scn1a +/+  | 21.6     | 23.7   | -2.1        | 28.3   |
|        | Scn1a +/KI | 22.5     | 23.8   | -1.3        | 27     |

transcription from the KI allele contains exon 20N, and that ~95% of exon 20N-containing transcripts are degraded, likely by nonsense-mediated decay.

As an alternative approach to evaluating usage of exon 20N, we constructed and analyzed RNA-seq libraries from brain tissue of four Scn1a +/KI mice and four Scn1a +/+ littermates. The number of reads that aligned to all Scn1a exons was 387.83 ± 56.49 and 223.24 ± 27.58 in +/+ and +/KI mice, respectively (Fig. 2C), but the number of reads that aligned to exon 20N were (0,0,1,0) and (0,0,0,0) in +/+ and +/KI mice, indicating that most transcripts that contain exon 20N are degraded.

**Figure 4. Increased inclusion of Exon 20N in Scn1a+KI brains.** (A) The positions of qPCR amplicons to quantify Scn1a mRNA transcripts. Amplicon 1 detects two isoforms (56bp and 120bp) of the Scn1a transcript, with the longer isoform reflecting exon 20N inclusion. Amplicon 2 quantifies only the Exon 20N-containing transcript. Amplicon 3 quantifies the total Scn1a mRNA levels including the transcript with Exon 20N. (B) Bioanalyzer evaluation of RNA from Scn1a+/+ mouse brain amplified with amplicon 1, showing a single Scn1a peak at 56 bp. The peaks at 15-bp and 1500-bp are size markers recommended and supplied by the manufacturer. (C) Bioanalyzer evaluation of RNA from Scn1a+/+ mouse brain amplified with amplicon 1, showing a second peak at 120 bp, representing inclusion of exon 20N. The 120-bp amplicon containing the 64-bp exon 20N is denoted with a red asterisk. (D) Scn1a+/+ and Scn1a+/KI mice had increased levels of the exon 20N-containing Scn1a transcript, measured using amplicon 2. The levels of exon 20N transcript (amplicon 2) expressed as a percentage of the total Scn1a levels (amplicon 3) using the formula (amplicon 2 levels)/(amplicon 3 levels)*100. (n = 4, 11.64 ± 2.90 months, Student’s unpaired t-test, p = 2e-4). **p < 0.01.

Table 1. Scn1a expression in various tissues. Quantification of total Scn1a mRNA levels using amplicon “1” (Figure 3) in Scn1a +/+ and Scn1a +/KI mice (n=1). Low levels of Scn1a mRNA were expressed in lung, liver, kidney, and heart as evidenced by the high \(\Delta\)Ct values. Tbp was used to normalize Scn1a expression. UD = undetectable. Transcripts containing 20N (Amplicon “2” in Figure 3) were undetectable or barely detectable in tissues other than brain.
Expressed as a proportion of reads that align to exon 20N compared to all other exons, ~70% of Scn1a transcripts include 20N at E14.5, gradually decreasing to <10% by P30, and remaining minimal throughout adult life (Fig. 5A). This pattern is inversely correlated with the overall level of Scn1a mRNA, inferred from the total number of reads (Fig. 5A). Thus, as exon 20N usage decreased, more Scn1a mRNA was produced, consistent with the poison exon inclusion being used to reduce Scn1a levels during development. We used the same dataset to evaluate usage of poison exon 18N in Scn8a and observed a very similar pattern (Fig. 5B). This confirms the results of Meisler and colleagues [37], and suggests that poison exons for both sodium channel genes serve a similar function.

**Discussion**

Here, we generated a knock-in mouse model of an intronic variant, previously identified as a *de novo* mutation in a patient with DS, to explore its effects on Scn1a expression and function *in vivo*. Introduction of this variant led to a

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**Figure 5:** Inverse relationship between poison exon usage and expression of multiple sodium channels during mouse brain development. (A) Scn1a transcripts including exon 20N are highly expressed in the developing mouse brain and decrease dramatically after birth (aqua bars), with a corresponding developmental increase in Scn1a expression (blue bars). (B) The poison exon in Scn8a previously described by Plummer et al. (ref 38). Scn1a exon 20N and Scn8a exon 18N are 37.5% identical (57% in human), and the amino acid sequences shown at exon boundaries are identical between the two genes. The amino acid sequences shown are fully identical between mouse and human for both genes. (C) Scn8a transcripts including exon 18N are highly expressed in the developing mouse brain and decrease dramatically after birth (aqua bars), with a corresponding developmental increase in Scn8a expression (blue bars).
reduction in brain Scn1a mRNA and Na 1.1 protein levels, resulting in expression of DS-related phenotypes (Figs. 2–3, Supplementary File 1). Inclusion of poison exon 20N in adult brain from Scn1a /+ mice was ~1% and increased ~ fivefold in +/KI mice. Together with additional analyses and earlier work [37], our results suggest that poison exons in at least two neuronal sodium channels serve an important function in developmental regulation, suppressing expression of functional sodium channels until later stages of brain development. The mechanism is evolutionarily conserved and represents a previously unrecognized potential source of Mendelian disease.

Mutations in the SCN1A gene are the most common cause of DS, accounting for 80% of patients. While 20% of patients still do not have a definitive molecular diagnosis after exome sequencing, our previous results identified a number of variants in and around exon 20N in multiple patients with DS/DEE [2]. Our results provide compelling evidence for pathogenicity of the variant reported here and, by extension, additional variants that may enhance inclusion of exon 20N. Our in vivo finding that poison exon 20N inclusion leads to Scn1a loss of function explains how the phenotype of Scn1a+/KI mice mimics that of other DS models, since reduced Scn1a expression is the common feature underlying DS. Scn1a loss of function results in decreased expression of Na 1.1, a voltage gated sodium channel responsible for promoting electrical excitability of neurons [41]. Loss-of-function Scn1a mutations would decrease neuronal activity. Scn1a is predominantly expressed in inhibitory GABAerger interneurons [12], so DS mutations decrease activity of the inhibitory circuitry and resulting disinhibition contributes to seizure generation [41].

Since there is no disease-modifying therapy for DS patients, better understanding the molecular pathogenesis in different families is critical. In terms of the intrinsic variant we studied, there is a growing list of therapeutic strategies that target mechanisms related to alternative splicing. An RNA-based therapeutic triggering poison exon inclusion in DHX9, a gene involved in Ewing sarcoma, has been exploited to enhance the efficacy of chemotherapy in cancer patients [42]. Additionally, targeting RNA splicing of SMN2, haploinsufficiency of which leads to spinal muscular atrophy (SMA), has led to the development of the first FDA-approved drug to treat SMA [43]. The Scn1a+/KI mouse model we have developed based on patients with non-coding variants may provide support to assess poison exon 20N targeting therapeutics postnatally and other methods that may lead to an increased expression of Na 1.1.

In summary, our data indicate that poison exon inclusion is a conserved mechanism to suppress gene expression that is induced by an intrinsic mutation in SCN1A leading to DS. These findings deepen our understanding of the molecular genetic mechanisms leading to DS and provide a new mouse model for studying the effects of a novel intronic mutation. Further, confirmation of the relevance of poison exon inclusion to a Mendelian disorder, coupled to the observation that multiple genes are regulated by this mechanism during development, suggests that variation affecting poison exons may be more broadly relevant to human disease.

Materials and Methods

Conservation Assessment

GERP Analysis. The pre-mRNA diagram constructed using the R package ggbioma [44] [45] with coordinates obtained from the UCSC Genome Table Browser [46]. Conservation was analyzed across the region using GERP (Genomic Evolutionary Rate Profiling) conservation scores for each position [47]. Positive GERP scores reflect a high level of conservation, while negative GERP scores reflect a neutral rate of substitution at the region.

Cross-species conservation analysis. Multiple sequence alignments were retrieved from the Vertebrate Multiz Alignment & Conservation [48] (100 Species) track of the UCSC Genome Table Browser [46].

Animals. Mice were on a congenic C57BL/6J background. Mice were housed in a pathogen-free barrier facility on a 12-hour light/dark cycle with ad libitum access to water and food (NIH-31 Open Formula Diet, #7917, Harlan). Mice were genotyped by PCR from tail tissue collected at weaning and at death. Both male and female mice were used in experiments. Littermate siblings were used as controls in each experiment. Experiments were completed by blinded investigators. For postmortem analyses, mice were anesthetized by Fatal-plus (Vortech) and perfused with 0.9 % saline. Tissues were then removed, weighed, and dissected for processing as described above. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

Targeted CRISPR Scn1a +/-KI Generation. The CRISPR guide was generated using an online tool (http://crispr.mit.edu). A mouse carrying the NC_000068.7:g.66293870C>G (GRCh38.p6) mutation was generated at the University of Alabama at Birmingham (UAB) Transgenic & Genetically Engineered Models Core (TGEMs). The reagents used were from Integrated DNA Technologies (IDT), Inc., Coralville, Iowa: Alt-R™ S.p. Cas9 Nuclease 3NLS (Cat # 1074181), Alt-R™CRISPRtracrRNA(Cat#1072533),Alt-R™CRISPR crRNA (sequence: 5'-TTGCTCACAATTTGATGGGG-3'), single-stranded donor oligonucleotide (ssODN) (sequence: 5'-A*C*A*TAAGTCACAGTGCAAGGATTAAA GGTTAGCAAAGGGGTAAATACGATTCCATAATA AAGGGCTGAGGGGAGGAACCACCCGCTCACCACg C C A T C C A A G T G G A G C A G A G A T T A T G C T A

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conditions and the resulting supernatant (S2) was used to determine protein concentration using Bradford protein assay (Thermo Scientific, Pierce, Coomassie Plus (Bradford) Protein Assay, PI23238). Protein samples were prepared with 4x LDS (Life Technologies, NuPAGE LDS Sample Buffer (4X), NP0007) and 10x reducing agent (ThermoFisher, 10X Bolt Sample Reducing Agent, B0009), heated for 10 min at 70°C, then 10 µg were loaded and separated on 4–12% NuPage acrylamide gels (ThermoFisher, NuPAGE 10% Bis-Tris Midi Protein Gels, 26-well WG1203BOX) with NuPage MOPS running buffer for 1.5 h at constant 150 V. Next, proteins were transferred to Immunobilon-FL PVDF membranes (Fisher, Millipore, SLGV033RS) using NuPage transfer buffer transfer system overnight at constant 30 V. The membrane was blocked in 50% Li-Cor Odyssey buffer (Li-Cor, 927-40000) in tris-buffered saline with 0.1% Tween (TBS-T) blocking buffer for 1 hour at room temperature and incubated with the appropriate primary antibody. The specific primary antibodies were diluted in 50% Odyssey blocking buffer in TBS-T as follows: anti-SCN1A (Na 1.1) (Alomone Labs, ACS-001, 1:1,000, overnight), anti-SCN1A (Na,1.1) (Antibodies Incorporated, 75-023, 1:1,000, overnight), anti-GAPDH (Millipore, MAB374, 1:5,000, 1hr), anti-Actin (Cell Signaling, 4967S, 1:1,000, 1hr). After primary antibody treatment, membranes were washed three times in TBS-T followed by incubation for 1 hour with Alexa Fluor 700- or 800- conjugated goat antibodies specific for mouse immunoglobulin G (1:20,000, Li-COR). Membranes were then washed three times in TBS-T, followed by a single wash in TBS, imaged on the LI-COR Odyssey fluorescence imaging system, and quantified using Li-CPR Image Studio.

Behavioral Assessment. For all behavioral tests, experiments were carried out during light cycle at least one hour after the lights came on. All mice were transferred to testing room for acclimation at least one hour prior to experiments. Testing apparatuses were cleaned by 75% ethanol between experiments and disinfected by 2% chlorohexidine after experiments were finished each day. All mice were tested in all the behavioral tests in the same order. Investigators were blind to the genotype of individual mouse at the time of experiment.

Open Field. Each mouse was placed into the corner of an open field apparatus (Med Associates) and allowed to walk freely for 10 minutes. Total and minute by minute ambulatory distance, jumps, stereotypic behavior counts, and percent time in center of each mouse were determined using the manufacturer’s software.

Elevated Plus Maze. Elevated Plus Maze (Med Associates) has two open arms and two closed arms. Mice were placed in the hub of the maze and allowed to explore for five minutes. The time in each arm, as well as entrances to each arm, explorations, and head dips over the edge of the maze, were monitored by video tracking software (Med Associates).

Y Maze. The Y-Maze apparatus consisted of three 15-inch long, 3.5-inch wide and 5-inch high arms made of white opaque plexiglass placed on a table. Each mouse was placed into the hub and allowed to freely explore for 6 minutes, with video recording. An entry was defined as the center of mouse body extending 2 inches into an arm, using tracking software (CleverSys). The chronological order of entries into respective arms was determined. Each time the mouse entered all three arms successively (e.g. A-B-C or A-C-B) was considered a set. Percent alternation was calculated by dividing the number of sets by the total number of entries minus two (since the first two entries cannot meet criteria for a set). Mice with 12 or fewer total entries were excluded from spontaneous alternation calculations due to insufficient sample size.

Tube Test for Social Dominance. The tube test for social dominance was conducted as previously described [31]. Mice of the same sex, but opposite genotype, were released into opposite ends of a clear plastic tube and allowed to freely interact. Under these conditions, one mouse will force the other out of the tube. The first mouse with two feet out of the tube was considered to have lost the match. Each mouse was paired with three different opponents of the opposite genotype, and the winning percentage was calculated for each mouse by dividing the number of wins by the total number of matches.

Three-Chamber Sociability Test. The three-chamber sociability test was conducted as previously described [32]. Mice were allowed to freely explore a three-chambered testing apparatus for 10 min prior the introduction of wire cages containing a novel mouse (adult sex-matched C57Bl/6J) or a novel object (Lego block). Investigation of the novel mouse and object was then monitored for 10 min using video tracking software (CleverSys).

Scn1a and Scn8a in Developing Mouse Brain. Publicly available RNA-seq data (SRA Accession # SRP055008) [40] was used to check the expression of Scn1a and Scn8a in the developing mouse cortex. The RNA-seq reads were trimmed using Trim Galore (https://github.com/FelixKrueger/TrimGalore). The trimmed reads were aligned to the mouse genome build GRCm38.p6 using STAR aligner. HTSeq was used to generate counts for genes from the alignments. Scn1a and Scn8a counts were extracted from the normalized table of counts in R package DESeq2. To calculate the proportion of 20N containing transcripts, samtools depth was used to extract coverage across each base of each exon of Scn1a and Scn8a [52]. The average read count per base was calculated by dividing the total read count by the size in bp of the exons. The averages for the 20N exon and all the exons were calculated separately. After normalizing to the number of mapped reads in each sample, percent poison exon usage was calculated using the formula: (average depth
of coverage of the poison exon) * (average depth of coverage of all exons) *100.

Statistics. mRNA and protein levels were analyzed by Student’s t-test. Behavioral tests were analyzed by Student’s t-test or two-way RM-ANOVA specified in the figures dependent on the outcome measure. The survival data were analyzed by Kaplan-Meier statistics and post-hoc Log-rank (Mantel-Cox) test.

Two-tailed p-values were calculated for all analyses, and the cut-off for statistical significance was set at 0.05. GraphPad Prism 7 was used for all analyses. Data are presented as mean ± SEM (Standard Error of the Mean). Significance denoted as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

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Figure S1. Multiple alignment of organisms with conservation in the SCN1A 20N region from the Multiz Alignment of 100 Vertebrates track from the UCSC Genome Browser.

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Figure S2. Full length Western blots of protein levels in Scn1a +/+ and Scn1a+/KI mouse brains. (A) Brain (frontal lobe) Scn1a protein levels in Scn1a+/KI vs. Scn1a+/+ mice using rabbit anti-Na\textsubscript{v} 1.1 (Scn1a) antibody from Alomone Labs. (B) GAPDH using anti-GAPDH antibody from Millipore was used as loading control. (C) Scn1a protein levels using anti-Na\textsubscript{v} 1.1 (SCN1A) UC-Davis antibody. (D) Actin using anti-Actin antibodies from Cell Signaling was used as loading control. (E) RNA-seq counts of Gapdh mRNA from DEseq2 analysis in whole brains of Scn1a+/KI and Scn1a Scn1a+/- mice (n = 4, 11.64 ± 2.90 months, Student’s unpaired t-test, p = 0.67).
**Figure S3.** *Scn1a*+/KI mice have no behavioral changes detected in the elevated plus and Y mazes. (A) Time spent in open arms of the elevated plus maze (*n* = 6–8, 13.81 ± 0.47 months, Student’s unpaired t-test, *p* = 0.1475). (B) Time spent in closed arms of the elevated plus maze (*n* = 6–8, 13.81 ± 0.47 months, Student’s unpaired t-test, *p* = 0.0958). (C) Total arm entries of the elevated plus maze (*n* = 6–8, 13.81 ± 0.47 months, Student’s unpaired t-test, *p* = 0.1577). (D) Correct alternations in the Y maze (*n* = 6–8, 13.81 ± 0.47 months, Student’s unpaired t-test, *p* = 0.5888). (E) Total distance travelled during 5 min in the Y maze (*n* = 6–8, 13.81 ± 0.47 months, Student’s unpaired t-test, *p* = 0.1242). All data are expressed as mean ±/– SEM.

**Figure S4.** *Scn1a*+/KI mice have no social behavior deficits detected. (A) *Scn1a*+/KI mice win equally to the littermate *Scn1a*+/+ mice in the social dominance tube test (*n* = 6–8, 13.81 ± 0.47 months, Student’s unpaired t-test, *p* > 0.9999). (B) During habituation, mice of both genotypes had no preference to the side (top or bottom) of the three-chamber box (*n* = 6–8, 13.81 ± 0.47 months, two-way RM-ANOVA, interaction *p* = 0.1484, main effect of side *p* = 0.04828, main effect of genotype *p* = 0.4806). (C) During testing, mice of both genotypes had no preference to a Lego block or a stranger mouse (S) as measured by time spent in a specific chamber containing a Lego block or a stranger mouse (S) as measured by time spent in a specific chamber containing a Lego block or a stranger mouse (*n* = 6–8, 13.81 ± 0.47 months, two-way RM-ANOVA, interaction *p* = 0.08994, main effect of stranger mouse *p* = 0.1339, main effect of genotype *p* = 0.2311). (D) *Scn1a*+/KI mice were not significantly different from *Scn1a*+/+ littermate controls in time spent around a cup containing stranger mouse compared to time spent around a cup containing a Lego object (*n* = 6–8, 13.81 ± 0.47 months, two-way RM-ANOVA, interaction *p* = 0.1969, main effect of stranger mouse *p* = 0.0167, main effect of genotype *p* = 0.0867). All data are expressed as mean ±/– SEM.