Received: 22 July 2020 | Revised: 5 October 2020 | Accepted: 6 October 2020

DOI: 10.1111/epi.16741

FULL LENGTH ORIGINAL RESEARCH

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Gabra2 is a genetic modifier of *Scn8a* encephalopathy in the mouse*

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Funding information
National Institutes of Health, Grant/Award Number: 5U42OD010924, NS034509, R01ES029925 and U24HG010100

Abstract

Objective: *SCN8A* encephalopathy is a developmental epileptic encephalopathy typically caused by de novo gain-of-function mutations in $Na_v1.6$. Severely affected individuals exhibit refractory seizures, developmental delay, cognitive disabilities, movement disorders, and elevated risk of sudden death. Patients with the identical *SCN8A* variant can differ in clinical course, suggesting a role for modifier genes in determining disease severity. The identification of genetic modifiers contributes to understanding disease pathogenesis and suggesting therapeutic interventions.

Methods: We generated F1 and F2 crosses between inbred mouse strains and mice carrying the human pathogenic variants *SCN8A*-R1872W and *SCN8A*-N1768D. Quantitative trait locus (QTL) analysis of seizure-related phenotypes was used for chromosomal mapping of modifier loci.

Results: In an F2 cross between strain SJL/J and C57BL/6J mice carrying the patient mutation R1872W, we identified a major QTL on chromosome 5 containing the *Gabra2* gene. Strain C57BL/6J carries a splice site mutation that reduces expression of *Gabra2*, encoding the α2 subunit of the aminobutyric acid type A receptor. The protective wild-type allele of *Gabra2* from strain SJL/J delays the age at seizure onset and extends life span of the *Scn8a* mutant mice. Additional *Scn8a* modifiers were observed in the F2 cross and in an F1 cross with strain C3HeB/FeJ.

Significance: These studies demonstrate that the SJL/J strain carries multiple modifiers with protective effects against seizures induced by gain-of-function mutations in *Scn8a*. Homozygosity for the hypomorphic variant of *Gabra2* in strain C57BL/6J is associated with early seizure onset and short life span. *GABRA2* is a potential therapeutic target for *SCN8A* encephalopathy.

KEYWORDS

epilepsy, epileptic encephalopathy, *Gabra2*, genetic modifier, *Scn8a*, voltage-gated sodium channel

*This article is dedicated to the memory of Kenneth and Beverly Paigen, geneticists *par excellence*.

1 | INTRODUCTION

Gene interactions play a major role in determining the severity of inherited disease. The different clinical course in patients with the same pathogenic disease variant is influenced both by differences in inherited genetic background and by stochastic variation in gene expression during normal development. Relevant genetic factors can be investigated by exploiting genetic variation between inbred strains of mice. Mapping specific genetic modifiers in crosses between strains can reveal basic biological mechanisms and suggest novel targets for therapeutic intervention.

SCN8A encephalopathy (Online Mendelian Inheritance in Man database 614558) is a developmental and epileptic encephalopathy (DEE) typically caused by de novo mutations of the neuronal sodium channel gene *SCN8A*.[1] Several hundred affected individuals have been identified, and the predominant molecular mechanism in severely affected individuals has been found to be gain-of-function (GOF) missense mutations resulting in altered biophysical properties and elevated channel activity. The average age at onset of *SCN8A* encephalopathy is 4 months, and the disease course includes developmental delay and drug-resistant seizures.[2,3] Approximately 50% of individuals are severely affected and remain nonambulatory. Intellectual disability as well as sleep and movement disorders are common comorbidities.[2,4–6] Increased early mortality is a feature of the syndrome.[7]

We previously generated two mouse models of *SCN8A* encephalopathy expressing patient variants, a constitutive knockin of the variant p.Asn1768Asp (N1768D)[8] and a conditional knockin of the variant p.Arg1872Trp (R1872W).[9,10] N1768D, the first identified mutant channel in this disorder, causes impaired channel inactivation and neuronal hyperactivity.[11] Mice expressing *Scn8a*-N1768D exhibit early onset, spontaneous convulsive seizures, and sudden death. R1872W is a recurrent mutation resulting from CpG deamination of the arginine codon CGG.[12] De novo mutations in this arginine codon have been identified in 21 unrelated patients.[7] The positively charged arginine residue 1872 is located in the cytoplasmic C-terminal domain of the channel and interacts with negatively charged residues in the inactivation gate of the channel.[13] Loss of this interaction destabilizes the inactive channel and leads to elevated channel activity.[9] Patients with the R1872W mutation exhibit infantile onset DEE, status epilepticus, severe motor disabilities, and sudden death.[3,7,14–16] Mice expressing *Scn8a*-R1872W in forebrain excitatory neurons under the control of Emx1-Cre demonstrate early onset convulsive seizures and juvenile lethality.[10]

To identify genetic modifiers of *SCN8A* encephalopathy, we carried out crosses between three inbred strains and C57BL/6J mice carrying the mutations N1768D and R1872W. Crosses with strain SJL/J delayed seizure onset and extended survival of the mutant mice. We describe the genetic mapping of one major modifier, a splice site variant in the *Gabra2* gene encoding the α2 subunit of the γ-aminobutyric acid type A ($GABA_A$) receptor. Mutations of human *GABRA2* have been identified in patients with generalized epilepsy with febrile seizures plus, absence epilepsy, and epileptic encephalopathy.[17–19] An excess of rare variants in the $GABA_A$ pathway was also observed in a large-scale genome sequence study of patients with epilepsy.[20] Recent studies suggest that the α2 subunit plays a critical role in inhibitory synaptogenesis.[21] Disruption of subcellular localization of the α2 subunit resulted in seizures and early mortality.[22] Strain C57BL/6J carries a hypomorphic splice site variant that reduces *Gabra2* transcript level by 75%.[23] The hypomorphic variant arose approximately 40 years ago at the Jackson Laboratory and is not found in the C57BL/6N subline.[23] We now report that the hypomorphic *Gabra2* variant cosegregates with early seizure onset and reduced life span in $Scn8a^{R1872W/+}$ mutant mice. This study provides the basis for future analysis of the interactive effect of human *GABRA2* variants on the severity of *SCN8A* encephalopathy.

Key Points

- Mouse strain SJL/J carries multiple genetic modifiers that delay seizure onset and extend survival of mice expressing the patient mutation p.*SCN8A*-Arg1872Trp
- QTL analysis of an F2 cross between strains C57BL/6J and SJL/J mapped a major modifier locus to chromosome 5 and identified several potential minor loci
- The hypomorphic *Gabra2* variant on chromosome 5 in strain C57BL/6J mice increases seizure severity, suggesting that upregulation of *Gabra2* could be therapeutic

2 | MATERIALS AND METHODS

2.1 | Animals

The conditional $Scn8a^{R1872W}$ allele contains two copies of the last coding exon of *Scn8a*, a floxed wild-type exon located upstream of an exon with the R1872W mutation.[10] The action of Cre recombinase deletes the wild-type exon from the conditional allele and activates the R1872W mutant. The conditional allele was generated by transcription activatorlike effector nuclease knockin to a (C57BL/6JX SJL/J)F2 zygote.[10] The conditional allele was inserted into the C57BL/6J-derived *Scn8a* locus on chromosome 15; the conditional line was bred from a single founder individual and maintained by >10

generations of backcrossing to strain C57BL/6J. The mutant line C3HeB/FeJ *Scn8a*^{N1768D} expresses the original *SCN8A* mutant, N1768D,[8] and was generated by >10 generations of backcrossing to strain C3HeB/FeJ.[24] C57BL/6J *Emx1*$^{Cre/Cre}$ mice with a *Cre* transgene inserted into the endogenous *Emx1* locus (Jackson Laboratory catalog # 005628), and inbred strains C3HeB/FeJ, FVB/NJ, and DBA/2J were obtained from the Jackson Laboratory. *Emx1*^{Cre} mice express Cre recombinase in forebrain excitatory neurons beginning at E9.5.[25,26] Mice carrying both the conditional *Scn8a*^{R1872W} mutation and the *Emx1*^{Cre} are designated conditional knockin (cKI) mice. All animal experiments were approved by the University of Michigan and the Unit for Laboratory Animal Medicine at the University of Michigan in accordance with the National Institutes of Health Guide for the Care and Use of Animals (protocol # PRO00007986). Principles outlined in the ARRIVE guidelines and the Basel declaration https://www.basel-declaration.org/ including the 3R concept were considered when planning the experiments.

2.2 | Genotyping

The conditional allele of *Scn8a* was genotyped by polymerase chain reaction (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. Digestion of the PCR products with KpnI generates an intact 414-bp fragment from the wild-type allele and fragments of 262 bp and 186 bp from the conditional allele. The presence of *Emx1*^{Cre} was detected by PCR amplification of a 102-bp product using forward primer 5′-GCGGT CTGGC AGTAA AAACT ATC-3′ and reverse primer 5′-GTGAA ACAGC ATTGC TGTCA C T-3′.

2.3 | Targeted deep sequencing of the *Scn8a* transcript

Total RNA was prepared from whole brain by TRIzol extraction using a Direct-zol RNA Miniprep Plus Kit (Zymogen R2071). cDNA was synthesized with the LunaScript RT SuperMix Kit (New England BioLabs). 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 PCR product was performed with the Illumina MiSeq system. Raw data were aligned to the mm10 reference genome using HISAT2[27] and analyzed using SAMtools mpileup.[28] The number of sequence reads varied from 1100 to 6749 per sample, with the exception of one sample with 339 reads.

2.4 | Quantitative RT-PCR of *Gabra2*

Hippocampus was dissected from adult mice and cDNA was prepared as described above. The abundance of the *Gabra2* transcript was assessed using the TaqMan gene expression assay (Mm01277042_m1) and normalized to *Tbp* as internal control (Mm01277042_m1). TaqMan assays were run on a StepOnePlus cycler (Applied Biosystems) using StepOne software v2.3 (Life Technologies). Relative transcript level was calculated by the $\Delta\Delta C_t$ method[29] and compared with C57BL/6J brain.

2.5 | Quantitative trait locus analysis

Genomic DNA was extracted from tail biopsy using GenElute Mammalian Genomic DNA Miniprep Kit (Sigma). Single nucleotide polymorphism (SNP) genotyping with the miniMUGA array[30] was carried out at NeogenCorp (Lincoln, NE). The MiniMUGA array is designed to discriminate between most commonly used mouse populations and contains probes for >10 000 SNPs. Of these, 2792 were found to robustly discriminate between strains C57BL/6J and SJL/J and were retained for analysis. To achieve approximate normality, a log transformation was applied to the age at death (survival) phenotype. Data analysis was carried out using R/qtl2.[31] Briefly, we fit a linear mixed model at each of the 2792 retained loci, regressing the (transformed) phenotype of interest on the allele probabilities. The models also included sex as a fixed effect, and a random effect to control for the overall genetic similarity between samples at analyzed loci. The models were fit using maximum likelihood. Analysis was carried out on 111 affected F2 mice, the 60 mice described in the experiments below plus 51 mice evaluated for length of survival only.

3 | RESULTS

3.1 | Backcrossing *Scn8a*^{R1872W} mice to strain C57BL/6J accelerates seizure onset

The conditional *Scn8a*^{R1872W} allele was generated on the (C57BL/6J × SJL/J)F2 strain background, and the F2 founder was backcrossed to strain C57BL/6J (B6). Mice from the N2 backcross generation were bred with B6.*Emx1*$^{Cre/Cre}$ mice to generate N3 mice. In these mice, the R1872W mutant allele is activated in excitatory forebrain neurons beginning at E9.5.[25,26] The *Scn8a*$^{R1872W/+}$, *Emx1*$^{Cre/+}$ N3 mice exhibit generalized seizures beginning at 1 month of age, with median survival of 46 days (Figure 1A).[10] With increasing generations of backcrossing to strain C57BL/6J, seizure onset occurs earlier and life span decreases. For example, in the N8 backcross generation, the median survival of mutant mice

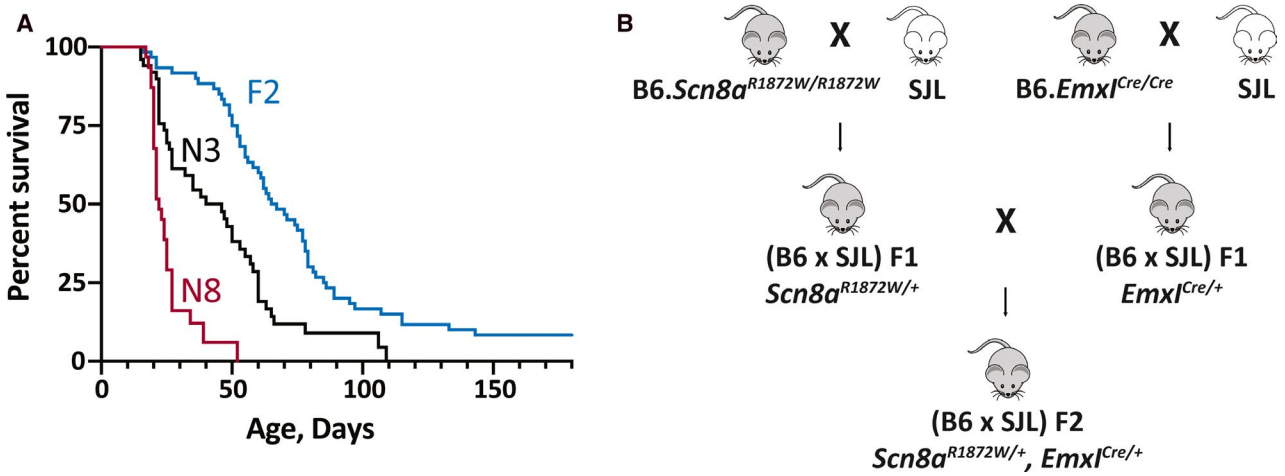

FIGURE 1 Strain background modifies survival of *Scn8a*$^{R1872W/+}$, *Emx1*$^{Cre/+}$ mice. The *Scn8a*-R1872W patient mutation was generated on the (C57BL/6J × SJL/J)F2 background and then backcrossed to strain C57BL/6J. A, Longer survival is correlated with a greater contribution of strain SJL/J. Approximate percentage SJL is 50% in the F2 mice, 6% in N3, and 0.2% in N8. B, Breeding scheme for double heterozygous F2 mice with genotype *Scn8a*$^{R1972W/+}$, *Emx1*$^{Cre/+}$. (N3 data are from previously published work[10])

was reduced to 22 days (Figure 1A). During backcrossing, the proportion of the genome derived from strain SJL/J is reduced from 50% in the F2 founder to an average of 0.2% in N8 mice, suggesting that loss of protective alleles from strain SJL/J might be responsible for the reduction in length of survival.

3.2 | Prolonged survival of (C57BL/6J × SJL/J)F2 mutant mice

To detect the presence of protective alleles in strain SJL/J, we crossed wild-type mice from this strain with N8 C57BL/6J *Scn8a*^{R1872W} mice to produce an F2 generation that was segregating alleles from both strains. The two-generation breeding scheme is shown in Figure 1B. In the first cross, C57BL/6J congenic homozygous *Scn8a*$^{R1872W/R1872W}$ mice from the N8 generation were bred with strain SJL/J to generate (B6 × SJL) F1 mice heterozygous for the conditional allele. In parallel, homozygous B6.*Emx1*$^{Cre/Cre}$ mice were crossed with strain SJL/J to generate (B6 × SJL)F1 mice heterozygous for *Emx1*^{Cre}. The two types of F1 mice were crossed to produce the F2 generation. In agreement with Mendelian prediction, *Scn8a*$^{R1872W/+}$, *Emx1*$^{Cre/+}$ double heterozygotes accounted for 60 of 248 F2 offspring. The median life span of the (B6 × SJL)F2 mutants was increased to 66 days (Figure 1A). The F2 mice with genotype *Scn8a*$^{R1872W/+}$, *Emx1*$^{Cre/+}$ are designated cKI, and these mice are analyzed in the following experiments.

3.3 | Seizure phenotypes in F2 cKI mice

The double heterozygous cKI F2 mice with genotype *Scn8a*$^{R8172W/+}$, *Emx1*$^{Cre/+}$ were monitored by observation for 8 hours per day, between 9 AM and 5 PM. Three to 4 days prior to onset of convulsive seizures, the mice developed hindlimb clasping, myoclonic jerks, and freezing behavior. Convulsive seizures began with 10-20 seconds of forelimb clonus with Straub tail followed by 5-15 seconds of violent clonic movements that propelled the mice around the cage (see Video S1). One to five seizures were observed per 8 hours of monitoring. Seizures occurred daily for 1-3 days after onset, followed by a seizure-free period of 2-14 days. A total of two to five cycles of seizures and seizure-free periods were observed. Death occurred overnight in 59 of 60 mice. Mice were found in the morning with extended body and limbs indicative of death during a convulsive seizure. Two mice displayed kyphotic postures.

3.4 | Age at seizure onset and length of survival in F2 cKI mice

N8 mice exhibit early seizure onset at 20-30 days and short survival (Figure) 2A,B. In contrast, the majority of F2 mice exhibited onset at 40-110 days of age (Figure 2C), with a broad distribution of life span from 20 days to >180 days (Figure 2D and Figure S1). At the extremes of the F2 distribution are five mice with survival of <30 days and five mice that are still surviving beyond 6 months of age.

There is a strong correlation between age at onset and length of survival in the F2 population (Figure 2E). There is little variation in duration of disease, the time between onset and death (Figure 3A). Duration was not correlated with onset, and the time for progression from seizure onset to death was similar in short-lived and long-lived mice (Figure 3B). The genetic variants segregating in this cross thus appear to primarily affect processes leading to

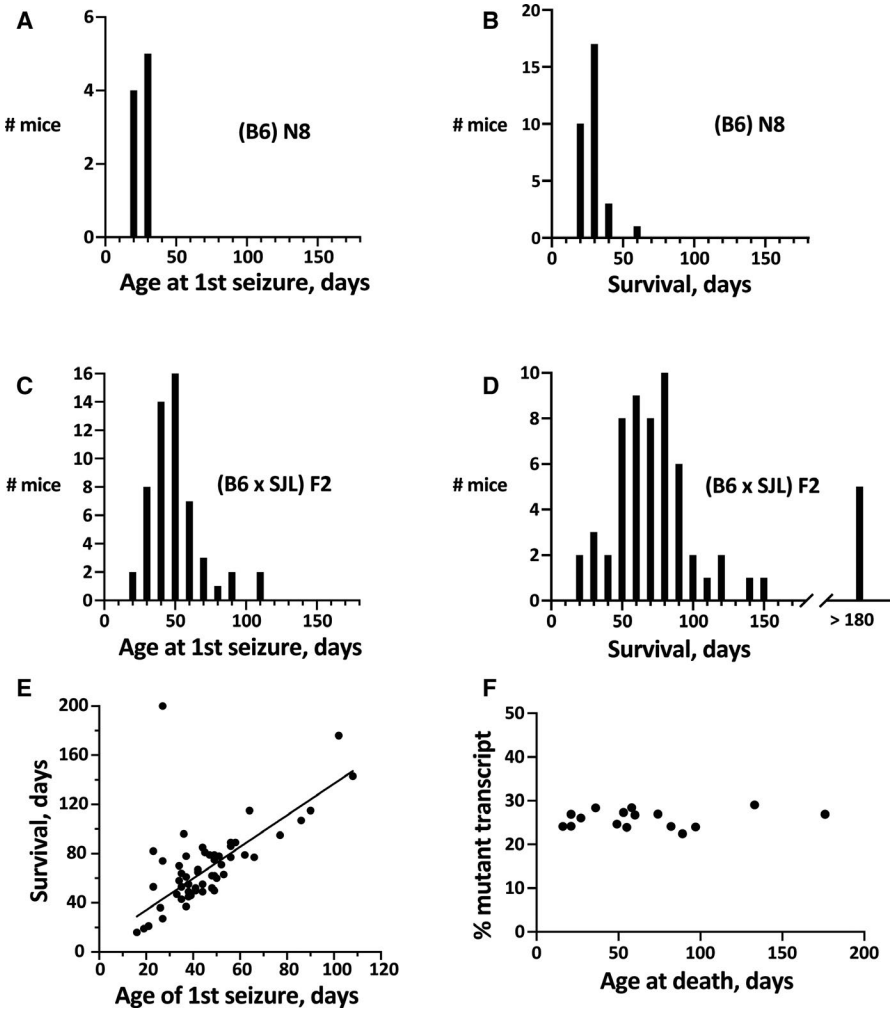

FIGURE 2 Seizure phenotypes in (B6 × SJL)F2 and C57BL/6J.N8 mutant mice. A, B, Distribution of age at seizure onset and length of survival in N8 mice. C, D, Distribution of age of onset and length of survival in F2 mice. See also Figure S1. E, Direct correlation between age at seizure onset and length of survival ($r^2 = 0.7$, n = 60; 1 outlier excluded from the calculation). F, In vivo activation of the conditional $Scn8a^{R1872W}$ allele in $Scn8a^{R1872W/+}$, $Emx1^{Cre/+}$ F2 mice. *Scn8a* transcripts were counted after deep sequencing of a reverse transcriptase polymerase chain reaction product containing codon1872. The proportion of mutant transcripts was 26 ± 2% (mean ± SD, n = 16) and did not differ between mice with early and late seizure onset

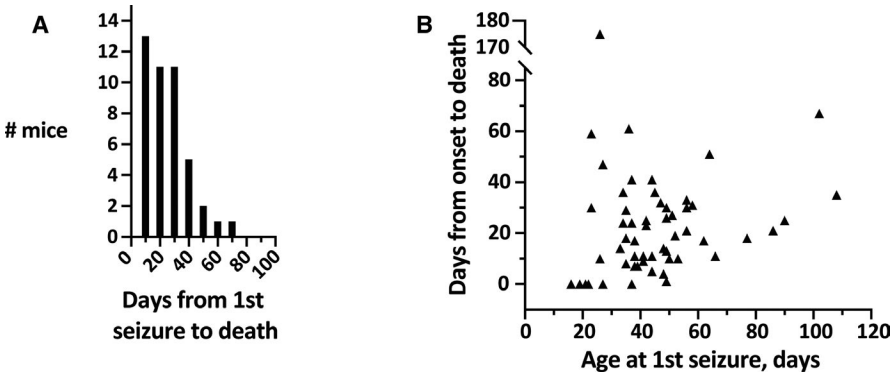

FIGURE 3 Disease duration is not correlated with age at onset of first seizure. Duration is defined as the time interval between first observed seizure and death. A, Narrow distribution of disease duration in (B6 × SJL)F2 mutant mice, from 20 to 40 days. B, Age at first seizure is not correlated with disease duration ($r^2 = 0.006$)

development of the first seizure, rather than progression from first seizure to death.

Activation of the conditional allele requires Cre-mediated deletion of exon 26a. The consistency of in vivo activation of the conditional allele was examined in F2 mice, with seizure onset varying between 20 and 180 days. An RT-PCR fragment containing codon 1872 was amplified from whole brain RNA, and the number of mutant and wild-type transcripts was determined by targeted deep sequencing. The proportion of transcripts containing the mutant sequence was 26 ± 2% and did not differ between mice surviving from 20 days to >100 days (Figure 2F). Long survival thus does not result from inefficient Cre-mediated activation of the conditional allele.

3.5 | Genetic mapping of loci affecting survival

Genetic mapping of the survival phenotype was performed on the 60 F2 mice with the cKI genotype $Scn8a^{R1872W/+}$,

Emx1$^{Cre/+}$ described above and 51 mice with the same genotype from a second F2 cohort comprised of subsequent litters from the same cross. A total of 111 F2 mice are included in the quantitative trait locus (QTL) analysis. The mapping results are presented as a Manhattan plot in Figure 4. The significance thresholds were derived using the empirical distributions of the lod scores based on 10 000 permutations. The 0.05 and 0.1 quantiles of these distributions are shown as black and red dotted lines, respectively. One major locus with a significant lod score ($P < .05$) was mapped to chromosome 5 (Figure 4).

In addition to the chromosome 5 QTL, two loci on chromosome 1 were significant at the .1 level, and a marginal peak was observed on chromosome 19. To further investigate the lod peaks that did not quite achieve significance at the .05 level, we also fit the models controlling for the allele probabilities associated with the peak on chromosome 5 as fixed effects; our conclusions did not change in this sensitivity analysis.

3.6 | *Gabra2*: A candidate gene in the chromosome 5 QTL

To define the maximum length of the QTL on chromosome 5, we examined the SNP haplotypes of the five mice with survival time of <30 days. These mice were homozygous for BB alleles in the interval that includes UNC9382069 at 67 Mb on chromosome 5 and S6R053314276 at 82 Mb. Within this interval, the *Gabra2* gene is located at 71 Mb. C57BL/6J and closely related strains are known to carry a single base deletion at position −3 in the splice acceptor site of exon 5 in *Gabra2*. Homozygosity for this variant reduces the abundance of the *Gabra2* transcript in hippocampus to 25% of wild type.[23] To determine whether the splice site variant is segregating in our F2 cross, we amplified genomic DNA from C57BL/6J and SJL/J mice from our colony and sequenced the *Gabra2* splice acceptor site. The thymine residue that is deleted in strain C57BL/6J is marked with an arrow above the SJL/J chromatograph in Figure 5A. The chromatograms identify the wild-type allele in strain SJL/J and confirm the deletion in strain C57BL/6J.

To determine whether the deletion is associated with a difference in *Gabra2* expression in the parental strains, we carried out RT-PCR of hippocampal RNA. The level of *Gabra2* transcript is 4.5-fold higher in strain SJL/J than in C57BL/6J (Figure 5B). This is consistent with other strains carrying the wild-type allele.[23,32]

To evaluate the physiological effect of the *Gabra2* variant, we examined the relationship between *Gabra2* genotype and seizure phenotypes. The median survival of *Gabra2*$^{B/B}$ mice was 53 days, significantly shorter than the 70 days and 79 days of BS and SS mice, respectively ($P < .05$; Figure 5C and Figure S2). The survival of BS and SS mice did not differ significantly ($P = .67$), demonstrating dominant expression of the wild-type Gabra2 allele (Figure S2).

Seizure onset was earlier in *Gabra2*$^{B/B}$homozygotes (median = 27 days) compared with BS and SS mice (median = 45 and 48 days, respectively; $P < .05$; Figure 5D and Figure S2). The difference between BS and SS mice was not significant ($P = .08$). These findings indicate that the hypomorphic *Gabra2* allele in strain C57BL/6J contributes to the more severe phenotype of earlier onset and shorter survival. The two *Gabra2*$^{B/B}$ mice exceeded 50 days, suggesting that these individuals inherited other protective loci from strain SJL/J. (the previous sentence should begin: "The age at seizure onset for two...")

3.7 | Additional protective modifiers in strain SJL/J

The congenic line C3HeB/FeJ *Scn8a*^{N1768D}[24] carries the patient mutation N1768D, which causes a defect in channel inactivation similar to that of R1872W with a less severe

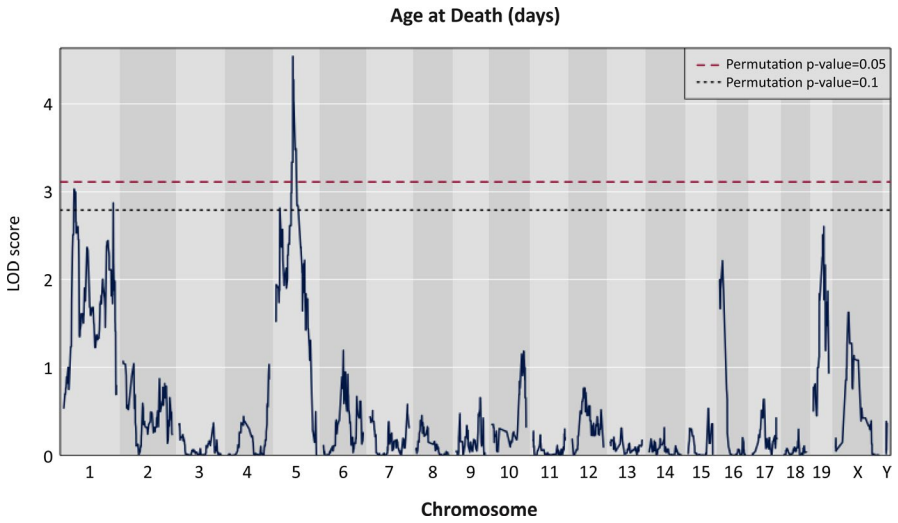

FIGURE 4 Quantitative trait locus mapping of survival in (B6 × SJL) F2 mice. One hundred eleven F2 mice were genotyped with the MiniMUGA genotyping array. Lod scores were calculated for 2792 informative loci. Chromosomes 1-19 are represented numerically on the x-axis. The relative width of the space allotted for each chromosome reflects the size of each chromosome. The y-axis represents the lod score

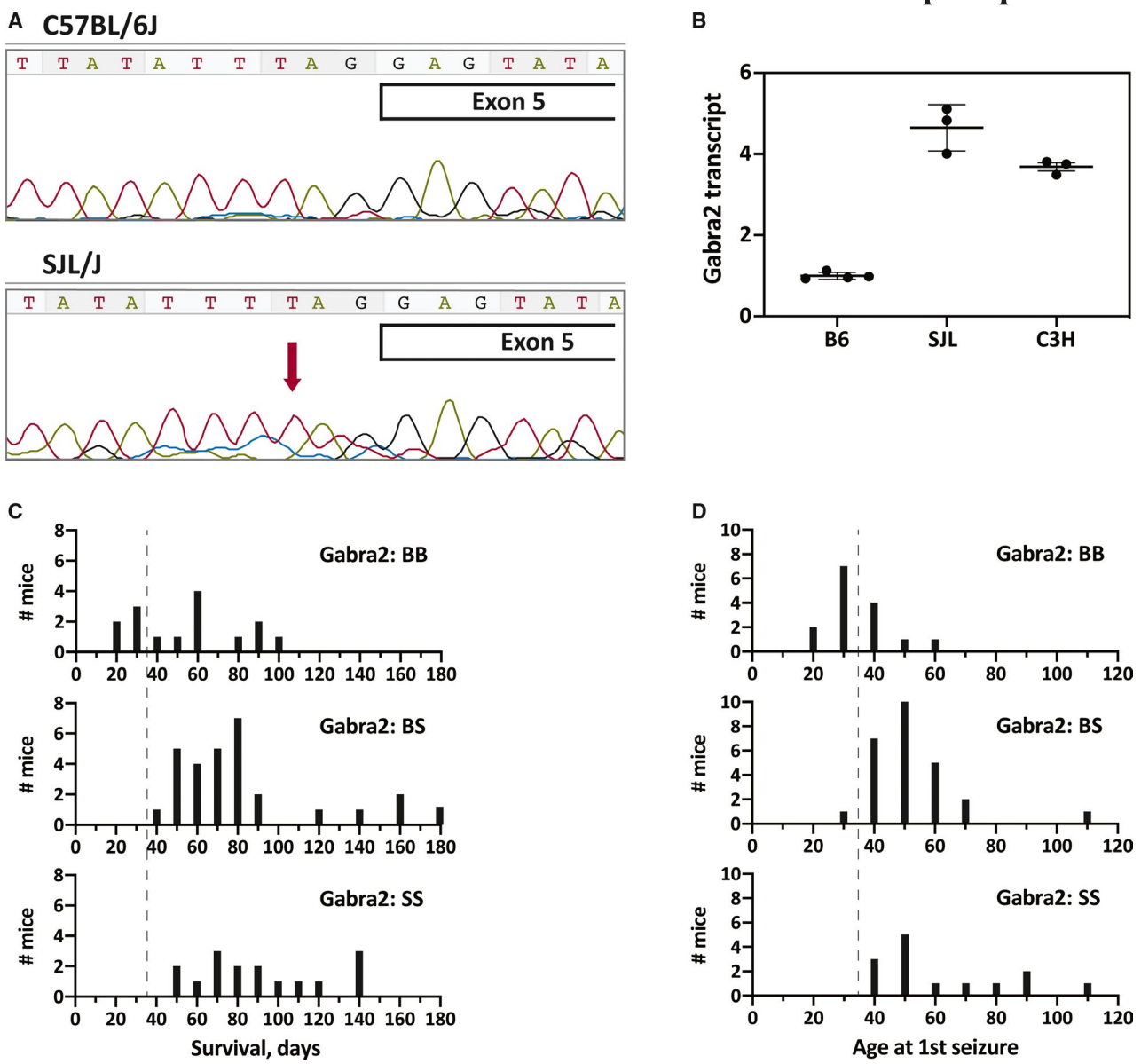

FIGURE 5 The *Gabra2* splice site variant cosegregates with seizure onset and survival in *Scn8a*$^{R1872W/+}$, *Emx1*^{Cre} F2 mice. A, SJL/J mice lack the splice site variant. A polymerase chain reaction product containing exon 5 was amplified from genomic DNA and sequenced. The T nucleotide in the wild-type allele at position −3 upstream of exon 5 is present in strain SJL/J (arrow). The single-bp deletion at position −3 (Mulligan et al[23]) is present in our C57BL/6J mice. B, The splice site variant reduces *Gabra2* transcripts. RNA was isolated from hippocampus of strains C57BL/6J, SJL/J, and C3Heb/FeJ, and *Gabra2* transcripts were quantitated with TaqMan assay. *Gabra2* transcripts are fourfold more abundant in strain SJL/J than in strain C57BL/6J. C, *Gabra2* genotype and length of survival in F2 mice. D, *Gabra2* genotype and age at first seizure in F2 mice. The dotted lines mark 35 days of surviva. See also Figure S2.

patient phenotype.[11,12] Strain C3HeB/FeJ does not carry the *Gabra2* splice site variant (Figure S3). The abundance of brain *Gabra2* transcript in strain C3H is comparable to strain SJL/J (Figure 5B). C3HeB/FeJ *Scn8a*$^{N1768D/+}$ mice exhibit 50% lethality at 6 months of age[24] (Figure 6A).

To confirm the presence of non-*Gabra2* modifier(s) in strain SJL, we tested the ability of SJL to rescue the N1768D *Scn8a* variant on the C3H strain background. In the (C3H × SJL)F1 cross, both parental strains contribute a wild-type *Gabra2* allele. Most of the (C3H × SJL) F1-*Scn8a*$^{N1768D/+}$ mice survived beyond 6 months (Figure 6A). These data demonstrate that strain SJL/J carries one or more protective modifier(s) other than *Gabra2,* and that these modifier(s) protect the N1768D allele of *Scn8a*.

To determine whether the protective modifier(s) are present in other inbred strains, we tested strains FVB/NJ and DBA/2J. These strains did not rescue the mutant mice in the cross with C3HeB/FeJ *Scn8a*$^{N1768D/+}$ (Figure 6B,C). Thus, the protective non-*Gabra2* modifier(s) that are present in strain SJL are not present in strains FVB/NJ and DBA/2J. This information will be useful for future identification of the additional modifier(s) in strain SJL.

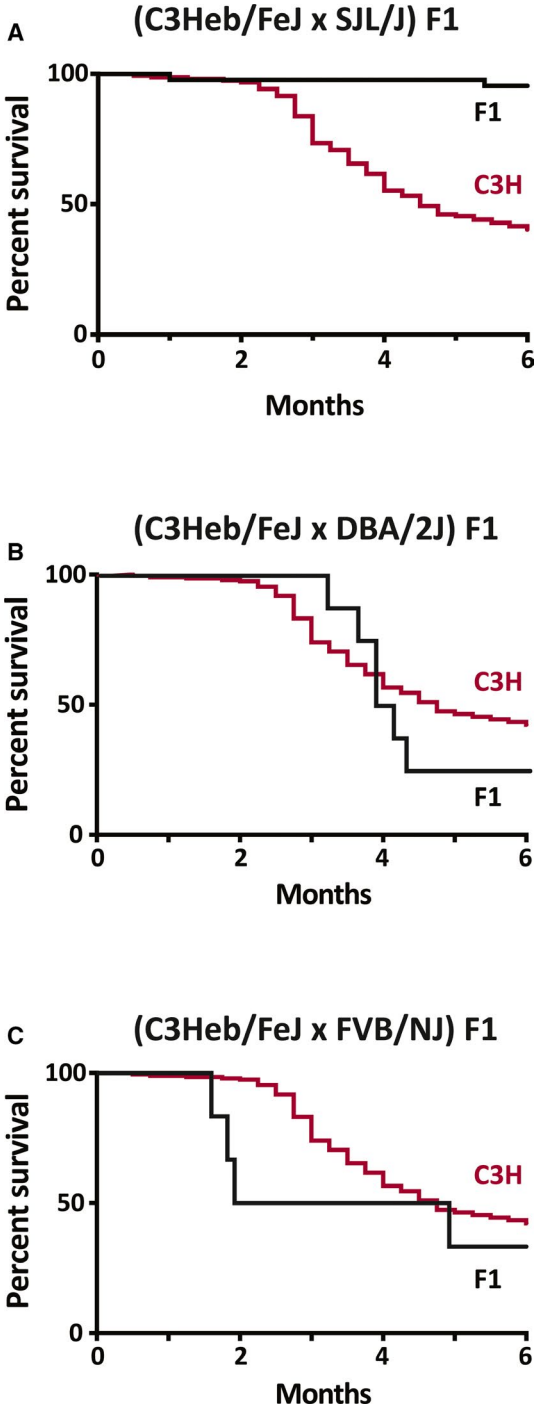

FIGURE 6 Survival of C3H *Scn8a*$^{N1768D/+}$ F1 mice. N20 mice from the congenic strain C3H *Scn8a*$^{N1768D/+}$ were crossed with inbred mice from strains SJL/J, FVB/NJ, and DBA/2J. Survival of F1 mice carrying the N1768D allele was monitored for 6 months. Survival was completely rescued in the (C3H × SJL)F1 mice

4 | DISCUSSION

Mice expressing the *Scn8a*-R1872W mutant channel in forebrain excitatory neurons exhibit a lethal seizure disorder with onset at 1-2 months of age and 100% penetrance.[10] We describe a modifier locus with an allele in strain SJL that significantly extends the survival of the *Scn8a* mutant mice. (C57BL/6J × SJL)F2 mice carrying the R1872W mutation exhibited a broad distribution of seizure onset, from 15 days to >6 months. QTL analysis confirmed the presence of a major segregating modifier locus on chromosome 5 and additional potential QTLs that did not reach significance in this cross.

The chromosome interval for the chromosome 5 modifier contains a gene previously implicated in seizure susceptibility. The GABA receptor α2 gene, *Gabra2*, encodes the α2 subunit of the receptor for the inhibitory neurotransmitter GABA. Strain C57BL/6J carries a hypomorphic variant of *Gabra2* that was first identified by its effect on exacerbation of seizures in *Scn1a* haploinsufficient mice.[33] The hypomorphic B6 allele was also found to confer sensitivity to flurothyl-induced seizures in the Collaborative Cross.[32] Mulligan et al[23] identified a splice site mutation in strain C57BL/6J that is responsible for reducing expression of *Gabra2* to 25% of wild-type levels. Correction of the single-bp deletion in strain C57BL/6J restored wild-type expression of *Gabra2*.

The work reported here extends the effect of the *Gabra2* hypomorph to GOF mutations of *Scn8a*. The segregation of the *Gabra2*splice site mutation in our cross was demonstrated by sequencing the C57BL/6J and SJL/J parental lines and genotyping the F2 offspring. The mutation was present in our colony of C57BL/6J mice, and strain SJL/J was found to carry the wild-type allele. We found that expression of the *Gabra2* transcript is 4.5-fold higher in strain SJL/J than in C57BL/6J. The splice site mutation segregated with early seizure onset and short survival in the F2 mice, demonstrating the modifier effect of *Gabra2* deficiency on seizures resulting from elevated activity of $Na_v1.6$. Physiologically, it appears that reduced GABAergic inhibition resulting from hypomorphic activity of GABRA2 exacerbates the elevated neuronal activity known to result from the GOF *SCN8A* mutations R1872W and N1768D.[9–11] Survival was shorter in *Gabra2*$^{B/B}$ mice but did not differ between heterozygous BS and homozygous SS, demonstrating recessive inheritance of susceptibility. The longer survival of some *Gabra2*$^{B/B}$ F2 mice suggested that additional protective modifiers are present in strain SJL/J, and this was confirmed in a cross with C3H *Scn8a*-N1768D mice. It will be of interest to determine whether patients with *SCN8A* encephalopathy carry variants in GABRA2 or other GABA receptor subunits that may be influencing the variable severity observed in individuals with *SCN8A* mutations.[3,16]

Gabra2 encodes the α2 subunit of the $GABA_A$ receptor, which is highly expressed in hippocampus, an important brain region in seizure generation.[34] The α2 subunit mediates localization of the $GABA_A$ receptor to axoaxonic synapses on the axon initial segment.[21,22,35] It is interesting that sodium

channel $Na_v1.6$, encoded by *SCN8A*, is also concentrated at the axon initial segment.[36–38] $GABA_A$ receptors containing α2 subunits could perhaps function locally to counter the elevated activity of GOF variants of *SCN8A*. Increased GABAergic activity might then be therapeutic for individuals with *SCN8A* GOF mutations. The antiepileptic drug clobazam is an activator of GABAergic neurotransmission that is used as an add-on drug for treatment of severe seizure episodes in *SCN8A* patients. AZD7325, a new positive allosteric modulator of α2 and α3 containing $GABA_A$ receptors, is less effective in mice with hypomorphic expression of *GABRA2*.[39] It is interesting that haploinsufficiency of *GABRA2* is underrepresented in the gnomAD population database,[40] with a calculated probability of loss-of-function intolerance = 1.0 and oe (observed/expected) = 0.05. It is also relevant that missense variants of 19 $GAGA_A$ receptor genes were associated with epilepsy risk in a whole exome sequencing study of 17 606 individuals.[20] The interaction of population variants of *GABRA2* with pathogenic variants of monogenic epilepsy genes will be an important area for future research. Future studies of neurological function in the mouse using strain C57BL/6J should take into consideration the potential impact of the hypomorphic variant of *Gabra2*.

ACKNOWLEDGMENTS

We thank Jacob Kitzman and Bala Burugula for NextGen sequencing of brain transcripts and Ben Pejsar of NeogenCorp for assistance with data transfer. This study was supported by National Institutes of Health NS 34509 (W.Y., S.F.H., M.H.M.) and 5U42OD010924, R01ES029925, and U24HG010100 (F.P.-M.d.V., J.G.X.).

CONFLICT OF INTEREST

None of the authors has any conflict of interest to disclose.

ETHICAL PUBLICATION STATEMENT

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Yu W, Hill SF, Xenakis JG, Pardo-Manuel de Villena F, Wagnon JL, Meisler MH. *Gabra2* is a genetic modifier of *Scn8a* encephalopathy in the mouse. *Epilepsia*. 2020;61:2847–2856. https://doi.org/10.1111/epi.16741