Confirmation of an Epilepsy Modifier Locus on Mouse Chromosome 11 and Candidate Gene Analysis by RNA-Seq

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Abstract

Epilepsy is a neurological disorder affecting approximately 1% of the worldwide population. Mutations in voltage-gated sodium channels have been identified in several monogenic epilepsy syndromes. Over 800 mutations have been identified in the voltage-gated sodium channel genes *SCN1A* and *SCN2A* in human epilepsies, including Genetic Epilepsy with Febrile Seizures Plus (GEFS+) and Dravet Syndrome. In GEFS+ families, affected members with the same mutation often display variability in clinical severity of the disease. This suggests that additional genes modify the effect of the primary mutation, resulting in the variable clinical presentation. The *Scn2a*^{Q54} transgenic mouse model has an epilepsy phenotype that varies depending on the genetic strain background. *Scn2a*^{Q54} mice congenic on the C57BL/6J strain exhibit delayed seizure onset and improved survival compared to (C57BL/6J × SJL/J)F1.Q54 mice. Two modifier loci of *Scn2a*^{Q54} seizure susceptibility were mapped and designated *Moe1* (modifier of epilepsy) on chromosome 11 and *Moe2* on chromosome 19. To confirm *Moe1* and refine its position, we generated interval specific congenic (ISC) lines carrying C57BL/6J-derived chromosome 11 alleles on the SJL/J strain and refined the map position to 89–104 Mb. We then used RNA-Seq for candidate analysis in the modifier region. C57BL/6J and SJL/J male and female brain RNAs were sequenced, revealing numerous significant transcriptome differences and coding SNPs. Additional consideration of gene function and expression suggested several strong candidate modifier genes, including two voltage-gated calcium channel subunits, *Cacna1g* and *Cacnb1*, and the PAR bZIP transcription factor, *Hlf*.

Keywords

genetic modifier; seizures; epilepsy; voltage-gated sodium channels; voltage-gated ion channels; mouse models

Introduction

Mutations in voltage-gated sodium channels are responsible for several types of human epilepsy (Meisler & Kearney 2005). Mutations in *SCN1A* were first identified in patients with Genetic Epilepsy with Febrile Seizures Plus (GEFS+) (Escayg et al. 2000). Subsequently, *SCN1A* mutations were identified in patients with Dravet Syndrome (DS), a severe infant-onset epileptic encephalopathy (Claes et al. 2001). To date, over 800 epilepsy mutations in *SCN1A* have been reported. A smaller number of mutations in *SCN2A* have been identified in patients with Benign Familial Neonatal-Infantile Seizures (BFNIS), GEFS+ and DS (Meisler et al. 2010). Affected family members with the same sodium channel

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mutation often display variability in clinical severity of the disease, a common feature of genetic epilepsy. This suggests that epilepsy phenotype is influenced by other factors, which may include genetic modifiers.

A common feature of mouse seizure models, including sodium channel mutants, is that seizure susceptibility and severity vary significantly depending on the genetic strain background. This indicates that genetic modifiers contribute to variable phenotype expression. These mouse models provide a useful system for identifying modifier genes that may also contribute to variable expressivity in human epilepsy patients. Genes that influence a mutant phenotype can be identified systematically by evaluating the seizure phenotype on different background strains. Several strain-dependent seizure susceptibility loci and genes have been identified (Chaix et al. 2007; Ferraro et al. 2001, 2004, 2007a, 2007b, 2010b, 2011; Frankel et al. 1995; Gershenfeld et al. 1999; Legare et al. 2000; Maihara et al. 2000; Winawer et al. 2011).

The transgenic mouse model $Scn2a^{Q54}$ has an epilepsy phenotype due to a mutation in $Scn2a$ that slows channel inactivation and results in persistent sodium current (Kearney et al. 2001). Severity of the epilepsy phenotype in $Scn2a^{Q54}$ mice is highly dependent on the genetic background. The phenotype is less severe on the resistant C57BL/6J (B6) strain, while it is more severe on the SJL/J (SJL) strain. We previously mapped two loci, $Moe1$ (Modifier of Epilepsy) on chromosome (chr) 11 and $Moe2$ on chr 19, that influence severity of the $Scn2a^{Q54}$ phenotype (Bergren et al. 2005). Fine mapping of $Moe2$ on chr 19 identified $Kcnv2$ as a strong candidate gene (Bergren et al. 2009). Transgenic transfer of the modified phenotype *in vivo* supported the identification of $Kcnv2$ as a modifier gene in mice. Discovery of two novel human $KCNV2$ mutations in pediatric epilepsy patients suggested that $KCNV2$ may also contribute to human epilepsy susceptibility (Jorge et al. 2011).

In the present study, we generated interval specific congenic (ISC) lines to confirm and fine map the $Moe1$ locus on chr 11. Our results indicated the $Moe1$ locus is complex with at least two modifiers in the interval that exhibit sex-specific effects. We then used an RNA-Seq approach for candidate gene analysis within the $Moe1$ sub-intervals. $Moe1$ RNA-Seq data revealed numerous transcriptome differences and coding SNPs between sex and strain. We further evaluated and prioritized candidate modifier genes that may contribute to strain-dependent differences in seizure susceptibility in $Scn2a^{Q54}$ mice.

Materials and methods

Mice

$Scn2a^{Q54}$ transgenic mice were generated as previously described (Kearney et al. 2001). The congenic line C57BL/6J.$Scn2a^{Q54}$ (B6.Q54) was established as described and is maintained by continued backcrossing of B6.Q54 hemizygous males to B6 females (Bergren et al. 2005). Mice were group-housed with access to food and water ad libitum. All studies were approved by the Vanderbilt University Animal Care and Use Committees in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Generation of ISC Lines

Four ISC lines were generated carrying B6-derived chr 11 segments in $Moe1$ on an SJL background. B6 males were crossed with SJL females to create F1 progeny which were then continually backcrossed to SJL to generate congenic lines. Whole genome genotyping was performed at generations N2 and N5 using the mouse MMDAP 768 SNP panel on the Illumina GoldenGate Platform (Moran et al. 2006). Animals retaining B6 alleles in $Moe1$ with the lowest percentage of B6 alleles in the rest of the genome were selected for breeding to the next generation. Once established, ISC strains were maintained by continued

backcrossing to SJL and genotyping for chr 11 markers at all generations. All ISC lines were backcrossed for 10 generations prior to experiments.

Genotyping

Mice were tail biopsied on postnatal day 14 and genotyped. DNA was prepared from tail biopsies by phenol:chloroform extraction and ethanol precipitation or using the Gentra Puregene Mouse Tail Kit according to the manufacturer's instructions (Qiagen). The $Scn2a^{Q54}$ transgene was genotyped as previously described (Bergren et al. 2005). ISC lines were genotyped for chr 11 microsatellite markers by PCR and analyzed on 2% agarose or 7% non-denaturing polyacrylamide gels.

Phenotyping

Chr 11 ISC females were crossed with hemizygous B6.Q54 males to generate (B6.Q54 × ISC.SJL/J).F1 offspring (abbreviated F1.Q54) carrying heterozygous or homozygous B6 alleles in *Moe1*. We used a similar phenotyping paradigm to that used for low-resolution mapping of the *Moe1* locus (Bergren et al. 2005). Briefly, F1.Q54 offspring underwent 30 minute video-taped observations at three and six weeks of age. All observation sessions occurred between 1:00 and 4:00 PM. Based on prior extensive video-EEG monitoring, spontaneous behavioral seizures were assessed offline by a blinded observer using Observer XT software (Noldus) (Kearney et al. 2001). We counted the number of visible focal motor seizures with forelimb clonus and repetitive movements lasting 1–5 seconds (Racine seizure scale 3). Three and six week seizure counts were combined to obtain a frequency of seizures in 60 minutes for each animal.

Statistical Analysis

Average seizure frequencies were compared between groups by ANOVA with Fisher's PLSD post-hoc tests (n 9 per group). Preliminary analysis indicated that males and females differed within the strains. Therefore, male and female data were analyzed separately.

RNA Isolation

Total RNA was isolated from whole brains of six week old B6 or SJL male and female mice with the TRIzol reagent according to the manufacturer's instructions (Invitrogen). RNA integrity was assessed on an Agilent 2100 Bioanalyzer and all samples received RIN scores of 8.20. For each group, total RNAs from 4 mice were combined to generate a pooled sample.

Sample Preparation for RNA Seq

Starting with 3 µg of total RNA, samples underwent poly-A selection, chemical fragmentation, and first and second strand cDNA synthesis using the TruSeq RNA sample preparation kit (Illumina). Following second strand cDNA synthesis, samples were prepared for sequencing on the Illumina HiSeq 2000 platform in the Vanderbilt Genome Sciences Resource (http://gsr.vanderbilt.edu/) according to standard methods (Bentley et al. 2008). Two multiplexed lanes of 100 bp single-end sequencing were performed, resulting in 75 million mappable reads per lane.

RNA-Seq Data Analysis

Base calling and filtering of sequence reads were performed with the Illumina pipeline (Bentley et al. 2008). Subsequent analysis was performed using the Tuxedo Tools on the GALAXY open, web-based platform (Blankenberg et al. 2010; Giardine et al. 2005; Goecks et al. 2010). Tophat aligned RNA-Seq reads to the mm9 genome using Bowtie, allowing

reads that span exon-exon boundaries to be aligned to the correct genomic location. Following alignment, novel splice variants were identified (Langmead et al. 2009a, 2009b; Trapnell et al. 2009). Cufflinks assembled transcripts, estimated abundance in fragments per kilobase of exon per million fragments mapped (FPKM) and tested for differential expression and regulation. Cuffcompare combined Cufflinks transcripts across sex and strains. Cuffdiff tested for statistically significant differences in total gene and transcript expression, splicing, transcription start sites (TSS) and promoter usage (Roberts et al. 2011a, 2011b; Trapnell et al. 2010). Parameter settings for each analysis tool are detailed in Supplemental Table 1.

Variant calls were performed by the Vanderbilt Computational Genomics Core using the Genome Analysis Toolkit (GATK) for SNP discovery. Quality filtering retained SNPs with a Phred score of >Q20. Additional manual filtering removed the following: SNPs that were different between male and female of the same strain, heterozygous or had missing sample data. SNPs that were homozygous alternate from the reference (C57BL/6J) genome were retained. VCF files were uploaded to Ensembl's Variant Effect Predictor to reveal the functional consequences of identified variants (McLaren et al. 2010). Non-synonymous (NS) amino acid substitutions were uploaded to SIFT (http://sift.jcvi.org/) to predict the potential impact on protein function (Kumar et al. 2009).

Results

Interval Specific Congenics

We previously mapped the $Scn2a^{Q54}$ modifier $Moe1$ to mouse chr 11 with a peak at D11Mit289 (95 Mb) (Bergren et al. 2005). Inheritance of an SJL allele at this locus conferred increased seizure resistance, while homozygosity for B6 alleles conferred increased susceptibility. To confirm the initial mapping results and refine the $Moe1$ interval, we generated four ISC lines with varying B6-derived chr 11 segments covering the $Moe1$ 1 LOD support interval on an SJL background. The resulting ISC lines were: ISC-A (D11Mit33-D11Mit58, 81–104 Mb); ISC-B (D11Mit33-D11Mit122, 81–93 Mb); ISC-C (D11Mit122-D11Mit58, 93–104 Mb); ISC-D (D11Mit284-D11Mit58, 89–104 Mb) (Fig. 1A).

ISC Phenotyping

Chr 11 ISC females were crossed with B6.Q54 males to generate F1.Q54 offspring that were heterozygous or homozygous for B6 alleles in $Moe1$ (Fig. 1B). F1.Q54 genotypes were obtained at the expected Mendelian ratios. F1.Q54 offspring underwent 30 minute video-taped observations for visible spontaneous focal motor seizures at three and six weeks of age. Average seizure frequencies (# seizures/60 minutes) were compared between ISC groups carrying homozygous B6 alleles and controls carrying heterozygous alleles. Preliminary analysis indicated within strain sex differences suggesting the possibility of sex-specific modifier effects.

Male mice carrying homozygous B6 alleles in the ISC-D interval (89–104 Mb) exhibited more seizures than heterozygous control littermates, while males with homozygous B6 alleles in the ISC-C interval (93–104 Mb) showed no difference compared to heterozygous control littermates (Fig. 1C) (F(4,70)= 3.321, p< 0.015). This suggests that the small region of non-overlap between ISC-C and ISC-D (D-C; 89–93 Mb) is likely responsible for increased seizure frequency in males.

Female mice carrying homozygous B6 alleles in the ISC-C interval (93–104 Mb) exhibited more seizures than heterozygous control littermates (Fig. 1D) (F(4,70)= 2.601, p< 0.043). This suggests that B6 homozygosity in the distal $Moe1$ interval is permissive in females.

Surprisingly, females with homozygous B6 alleles in the ISC-D interval (89–104 Mb) showed no difference in seizure frequency compared to heterozygous control littermates. This suggests that B6 homozygosity in the small region of non-overlap between ISC-C and ISC-D (D-C; 89–93 Mb) may mask the effect of the distal permissive modifier in females.

In both sexes, B6 homozygosity in the large ISC-A interval (81–104 Mb) that spans the entire *Moe1* LOD support did not result in a significant difference in seizure frequency compared to heterozygous controls. This suggests that B6 homozygosity in the proximal region of the *Moe1* interval may mask the effect of more distal permissive modifiers in both males and females. However, B6 homozygosity in the small ISC-B proximal interval (81–93 Mb) alone does not significantly reduce seizure frequency compared to heterozygous controls.

Candidate Gene Analysis by RNA-Seq

Due to the complexity of the *Moe1* interval and the high gene density of chr 11, we performed sequence-based transcriptome analysis in order to identify candidate modifier genes in *Moe1*. Focused analysis of *Moe1* revealed numerous significant differences in total gene and transcript expression, TSS, alternate promoter usage and splicing (Supplemental Table 2). Our ISC results indicated that B6 homozygosity in the D-C interval (89–93 Mb) increased seizure frequency in males. Within the D-C interval, RNA-Seq identified significant gene or transcript expression differences between B6 and SJL males in three genes. Based on our ISC experiments, B6 homozygosity in the ISC-C interval (93–104 Mb) increased seizure frequency in females. In the ISC-C region, RNA-Seq analysis revealed significant gene or transcript expression differences between B6 and SJL females in 32 genes. A complete list of significant differences in the *Moe1* region by RNA-Seq is provided in Supplemental Table 2.

To further evaluate candidate genes, we performed SNP analysis to identify genes with coding sequence polymorphisms between B6 and SJL. In region D-C (89–93 Mb), which contains 26 known and predicted genes, we identified 25 SNPs, including two NS SNPs in one gene. In interval C (93–104 Mb), which contains 534 known and predicted genes, we identified 630 SNPs, including 29 NS SNPs in 22 genes. The complete list of NS coding polymorphisms in *Moe1* and the predicted impact on protein function are provided in Supplemental Table 3.

Following RNA-Seq analysis, we prioritized candidate genes based on the ISC mapping results, consideration of gene function, location and timing of expression and prior evidence of involvement in neuronal hyperexcitability (Table 1). Top candidate modifier genes were identified as *Hlf* in males and *Cacna1g* and *Cacnb1* in females.

In the D-C interval (89–93 Mb) that contains a male-specific modifier, there were significant expression differences between B6 and SJL males in two genes, *Hlf* (hepatic leukemia factor) and *Stxbp4* (syntaxin binding protein 4) and a processed pseudogene, *GM11512*. *Stxbp4* functions in the insulin receptor signaling pathway and glucose transport (Min et al. 1999). Currently, there is no evidence linking *Stxbp4* to neuronal hyperexcitability. *Hlf* is a member of the proline and acidic amino acid rich basic leucine zipper (PAR bZIP) transcription factor family which has previously been linked to epilepsy (Gachon et al. 2004). There was a significant between-strain *Hlf* transcript difference in males only, consistent with a male-specific effect (Fig. 2). Taken together, this suggests that *Hlf* is a strong candidate modifier gene in males (Table 1).

The ISC-C interval (93–104 Mb) which harbors a female-specific modifier contains numerous genes with expression differences between B6 and SJL females. Of the five genes

with a total gene expression difference between females, three are involved in basic cellular processes and two have unknown function. RNA-Seq identified 32 genes with significant differences in transcript expression. A large fraction of these genes are involved in basic cellular processes, with no direct evidence connecting them to neuronal hyperexcitability. There was a significant strain difference in transcript expression of *Slc25a39*, which is a member of the mitochondrial solute carrier family 25 (SLC25) (Haitina et al. 2006). A family-based study of epilepsy with suggestive linkage to chr 17q12-24 demonstrated differential expression of *SLC25A39* in affected family members (Siren et al. 2010). However, sequencing did not reveal a pathogenic variant in this gene. Based on its tenuous association with epilepsy, *Slc25a39* was retained as candidate modifier gene with lower priority (Table 1).

Cacna1g and *Cacnb1* emerged as top candidate modifier genes in females based on their direct connection to epilepsy and the strong association between ion channels and epilepsy. Human mutations in both genes have been identified in epilepsy patients (Escayg et al. 1998; Ohmori et al. 2008; Singh et al. 2007). Differences in five major *Cacna1g* transcripts were identified between B6 and SJL females (Fig. 3). These transcripts have known differences in gating, kinetics and voltage-dependencies (Chemin et al. 2001; Emerick et al. 2006; Monteil et al. 2000). There were also several significant differences in promoter usage, TSS and splice variants between sex and strain. A *Cacnb1* female-specific difference was identified between B6 and SJL in transcript *Cacnb1*-006, a processed transcript that is not translated (Fig. 4). There were also several significant differences in promoter usage, TSS and splice variants between sex and strain.

Discussion

Genetic modifier loci influencing the $Scn2a^{Q54}$ epilepsy phenotype were initially mapped to chrs 11 (*Moe1*) and 19 (*Moe2*) in a backcross between B6 and SJL strains (Bergren et al. 2005). We constructed ISC strains to confirm the presence of *Moe1* on chr 11 and refine the critical interval. Our results revealed that the interval likely contains multiple modifiers that contribute to phenotype severity and exhibit sex-specific effects. Similar to our results, previous studies of epilepsy QTL in polygenic mouse models have demonstrated that the substructure of QTL can be complex with multiple genes contributing to the low-resolution mapping peak (Legare et al. 2000; Legare & Frankel 2000).

In females, B6 homozygosity in the ISC-C interval (93–104 Mb) conferred increased seizure frequency, suggesting the presence of a permissive modifier gene in this interval. In males, increased seizure frequency was associated with B6 homozygosity in the ISC-D (89–104 Mb) interval but not in the ISC-C interval (93–104 Mb). This suggests that a male-specific permissive modifier is located in the small non-overlapping interval between ISC-C and ISC-D (D-C; 89–93 Mb). Within the *Moe1* interval we identified a significant number of transcripts that exhibited within-strain sex differences. These differences likely result from multiple different causes, which may include brain sexual dimorphism, epigenetics and endocrine influences.

RNA-Seq analysis identified several strong candidate modifiers that may contribute to severity of the seizure phenotype, including *Cacna1g* and *Cacnb1* in females and *Hlf* in males. Although we identified significant transcript differences in these top candidate modifier genes, we did not identify SNPs within the coding regions. It is likely that non-coding SNPs contribute to the observed transcript differences. Future sequencing of the non-coding regions of *Cacna1g*, *Cacnb1* and *Hlf* will determine the contribution of non-coding SNPs.

Cacna1g is a T-type Ca^{2+} channel α_1 subunit which propagates low-voltage activated (LVA) Ca^{2+} currents (Ernst & Noebels 2009). T-type channels regulate neuronal firing by conducting Ca^{2+} during action potentials and can switch neurons between firing modes (Cain & Snutch 2010). T-type channels are expressed at high densities on thalamic neurons. These neurons innately oscillate during sleep, but can also inappropriately oscillate and cause a generalized seizure (McCormick & Contreras 2001; Perez-Reyes 2003). Rapid activation of T-type channels is responsible for the generation of low threshold spikes, which trigger epileptic "burst-firing" patterns. It is also responsible for abnormal spike-wave discharges (SWDs) in absence epilepsy that arise from synchronous firing of thalamocortical networks (Blumenfeld 2005; Contreras 2006; Ernst et al. 2009). Several spontaneous mouse models of absence epilepsy, including *lethargic*, *tottering* and *stargazer*, exhibit abnormalities in LVA Ca^{2+} currents, likely resulting in abnormal thalamocortical synchronization and absence epilepsy in the mutant animals (Zhang et al. 2002). Deletion of *Cacna1g* in mice suppressed $GABA_B$ receptor agonist-induced SWDs, indicating an essential role for this gene in seizure expression (Ernst et al. 2009; Kim et al. 2001). In humans, SNPs in *CACNA1G* have been associated with idiopathic generalized epilepsy (Singh et al. 2007).

Our RNA-Seq analysis identified expression, TSS, promoter usage and splicing differences in *Cacna1g* between sex and strain. Alternative splicing of T-type Ca^{2+} channels is known to contribute to their functional diversity. We identified significant strain differences between females in the expression of five major *Cacna1g* transcripts with known functional diversity. The inclusion of cassette exon 14 has been shown to shift steady-state inactivation in the hyperpolarized direction and accelerate activation and inactivation kinetics (Chemin et al. 2001; Emerick et al. 2006). Inclusion of cassette exon 26 contributes to more hyperpolarized steady state inactivation and slower recovery from inactivation (Chemin et al. 2001). Exon 25 has an internal donor site, resulting in a short exon 25 (B) or full length (A) (Chemin et al. 2001; Emerick et al. 2006; Mittman et al. 1999; Monteil et al. 2000). Transcripts with the 25A exon activate and inactivate at more hyperpolarized potentials, while 25B exon transcripts have slower activation and inactivation kinetics (Chemin et al. 2001). Based on this information, transcripts that exhibit significantly higher expression in SJL females have the most hyperpolarized steady-state inactivation potential and the fastest activation and inactivation kinetics (Fig. 3). We hypothesize that SJL females have a higher portion of *Cacna1g* channels that activate quickly, but inactivate rapidly and prematurely. This early inactivation state may lead to a low probability for neurons to reach synchronous oscillations, a common feature of epilepsy (Blumenfeld 2005; Contreras 2006; Ernst et al. 2009). Conversely, transcripts expressed at higher levels in B6 females compared to SJL are those with depolarized steady-state inactivation potentials and slower rates of activation and inactivation (Chemin et al. 2001) (Fig. 3). We hypothesize B6 females have an increased number of *Cacna1g* channels that are prone to hyperexcitability. This hyperexcitability emerges from an altered window current, resulting from inactivation shifts and rate changes, a known contributor to epilepsy (Vreugdenhil et al. 1998).

Cacnb1 encodes one of four homologous Ca^{2+} channel β subunits. β subunits modulate α_1 channel trafficking, gating and kinetics (Buraei & Yang 2010; Dolphin 2003; Lie et al. 1999). *Cacnb1* has four splice variants, which are highly expressed in the hippocampus and dentate gyrus (Buraei & Yang 2010; Dolphin 2003). β subunits have been previously linked to epilepsy in mice. *Lethargic* mice have a mutation in *Cacnb4* that results in absence epilepsy, severe ataxia and loss of motor coordination (Burgess et al. 1997; Escayg et al. 1998). In humans, surgical resection samples from temporal lobe epilepsy patients exhibited an up-regulation of *CACNB1* and *CACNB2* (Lie et al. 1999). It was hypothesized that the increased expression may underlie enhanced Ca^{2+} currents commonly seen in rodent kindling and kainate models of temporal lobe epilepsy (Beck et al. 1998; Faas et al. 1996;

Lie et al. 1999; Vreugdenhil & Wadman 1994). Several *CACNB1* mutations have been identified in patients with a history of febrile seizures, juvenile myoclonic epilepsy, episodic ataxia, generalized epilepsy and praxis-induced seizures (Escayg et al. 1998; Ohmori et al. 2008).

RNA-Seq analysis determined that SJL females express significantly higher levels of transcript *Cacnb1*-006 than B6 females (Fig. 4). Although this transcript does not contain an open reading frame, it does undergo post-transcriptional processing, suggesting the possibility that it may act as a long non-coding RNA (lncRNA). Recent evidence demonstrates the importance of non-coding RNAs (ncRNA) in the brain (Berretta & Morillon 2009; Brosius 2005; Cao et al. 2006; Kapranov et al. 2007; Mehler & Mattick 2006). Contribution of ncRNAs has been demonstrated for neural differentiation and cell identity, hippocampal development, oligodendrocyte myelination and synaptic plasticity (Qureshi et al. 2010). Additionally, lncRNAs have been implicated in neurodevelopmental, neurodegenerative, neuroimmunological and psychiatric disorders and, relevant to our studies, epilepsy (Qureshi et al. 2010). Mice deficient in the lncRNA *Evf2* had reduced numbers of GABAergic interneurons in the hippocampal formation and displayed reduced synaptic inhibition (Bond et al. 2009). This suggests the importance of a lncRNA in the formation of GABA circuitry and suggests a potential mechanism for hyperexcitability. The lncRNA *BC1* has also been implicated in a seizure phenotype. $BC1^{-/-}$ homozygous knockout mice exhibited audiogenic seizures and it is hypothesized that *BC1* lncRNA modulates hyperexcitability via the metabotropic glutamate receptor pathway (Zhong et al. 2009). It is possible that the *Cacnb1*-006 transcript may act as a lncRNA that directly or indirectly influences neuronal excitability.

Hlf is a member of the PAR bZIP transcription factor family. The loss of the PAR bZIP transcription factor family (*Hlf, Dbp, Tef*) results in mice with spontaneous, generalized tonic-clonic and absence seizures (Gachon et al. 2004). Microarray expression analysis performed to investigate contributors to the PAR bZIP knockout epilepsy phenotype, revealed a two-fold reduction in pyridoxal kinase (*Pdxk*), which encodes a coenzyme involved in the conversion of vitamin B6 to pyridoxal 5′-phosphate (PLP) (Gachon et al. 2004). PLP is a key coenzyme involved in amino acid and neurotransmitter metabolism (John 1995). Mice defective in PLP metabolism experience fatal seizures, found to be a consequence of significantly reduced GABA levels (Waymire et al. 1995). Mice maintained on a vitamin B6 deficient diet, resulting in reduced PLP levels, experienced increased audiogenic seizures (Coleman & Schlesinger 1965; Schlesinger R & Schreiber 1969; Schlesinger & Uphouse 1972). Human vitamin B6-dependent epilepsies can be successfully treated with PLP (Plecko & Stockler 2009). Our RNA-Seq analysis showed that B6 males have a significant reduction in *Hlf* expression compared to SJL males. Reduced expression of *Hlf* in B6 males may lead to reduced Pdxk and PLP levels and increased seizure susceptibility. Future studies testing the effect of vitamin B6 deficiency on B6.Q54 mice may help determine the contribution of this pathway to $Scn2a^{Q54}$ epilepsy severity.

Based on our ISC and RNA-Seq data, along with further analysis of gene function and association with seizures, we nominate *Cacna1g*, *Cacnb1* and *Hlf* as strong functional candidates for *Moe1*. Future testing of high priority candidate genes will be necessary to determine if they contribute to the epilepsy severity of $Scn2a^{Q54}$ mice. Understanding the molecular basis of genetic modifiers in a mouse model can provide insight into human epilepsy. It has the potential to advance molecular diagnostic capabilities and identify novel therapeutic targets for the improved treatment of human patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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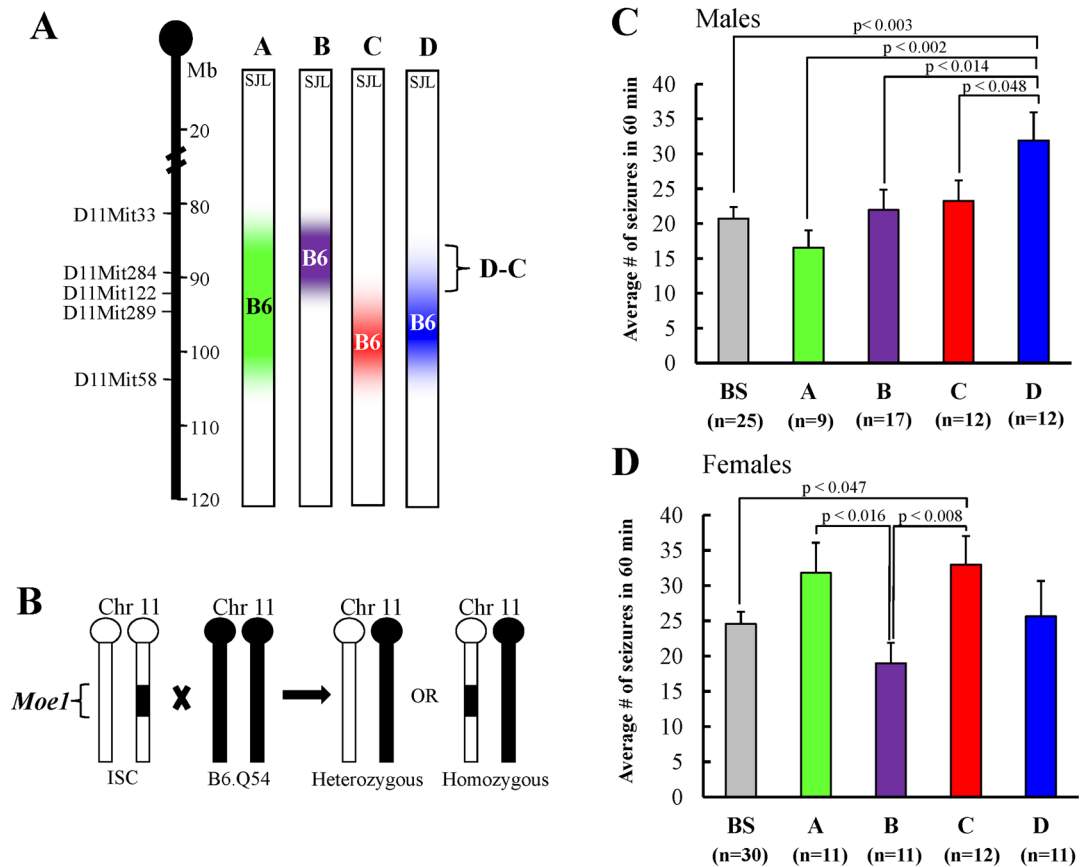
Figure 1. Fine mapping of Moe1 on chr11 with interval specific congenic lines
A. ISC lines were generated with varying B6-derived chr 11 segments (colors) on an SJL background (white). Microsatellite markers used for ISC generation are identified along the chromosome. From the original mapping, the *Moe1* peak was at D11Mit289. **B.** ISC lines were crossed with B6.Q54, generating F1.Q54 offspring. F1.Q54 mice will be heterozygous or homozygous for B6 alleles in *Moe1*. **C.** Seizure frequency in ISC males. Average number of seizures in 60 minutes for each genotype group is shown. Significant differences between groups were determined by ANOVA with Fishers PLSD post-hoc tests. **D.** Seizure frequency in ISC females. Average number of seizures in 60 minutes for each group is shown. Significant differences between groups were determined by ANOVA with Fishers PLSD post-hoc tests. Data are represented as mean ± SEM. Significant P-values determined by post-hoc Fishers PLSD are indicated in the figure.
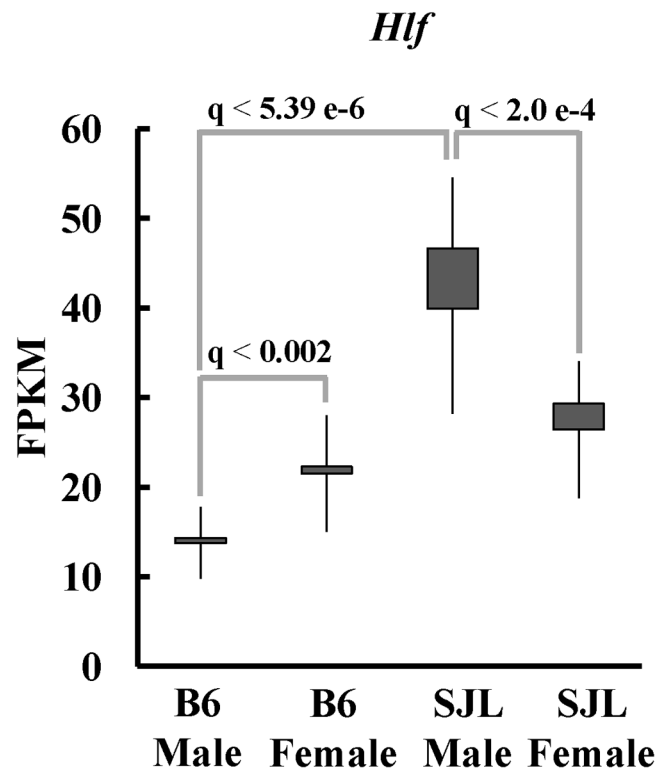
Figure 2. Expression of Hlf-002 transcript in B6 and SJL brain
The RNA-Seq FPKM values and confidence interval for each group is shown. Boxes represent the range of values and whiskers represent the 95% confidence interval calculated by Cuffdiff. Q-values are false discovery rate-adjusted p-values. (*Hlf*-002=ENSMUST00000043658).
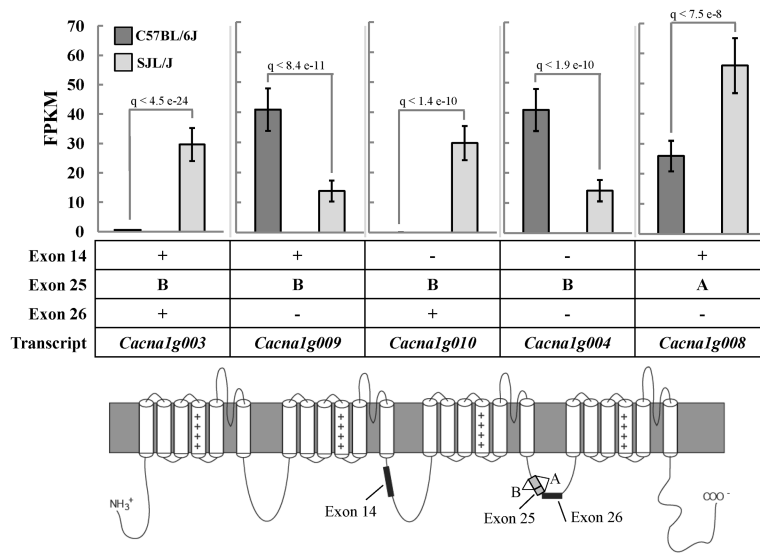
Figure 3. Differential expression of Cacna1g alternatively spliced transcripts in females
Inclusion/exclusion of cassette exons 14 and 26 and alternate splicing of exon 25 are shown
for the five transcripts that exhibited significant differences between B6 and SJL females.
The location of alternatively spliced exons are shown on the *Cacna1g* channel protein
(bottom). FPKM values and confidence intervals for B6 and SJL females are shown. Q-
values are false discovery rate-adjusted p-values.
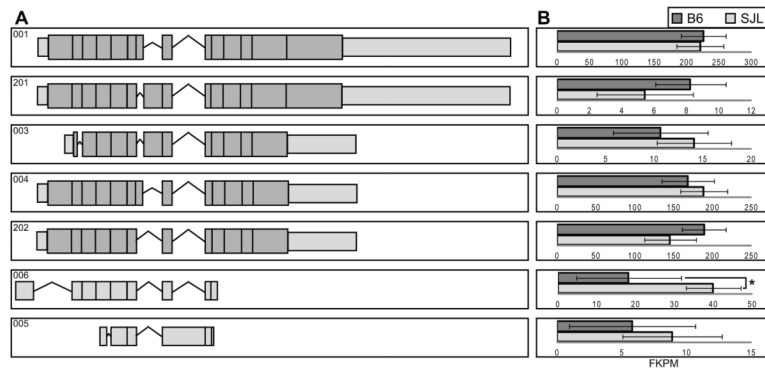
Figure 4. Alternative splicing and differential expression of Cacnbl
A. *Cacnb1* alternatively spliced transcripts are shown. Dark shaded boxes represent coding regions and light shaded boxes are non-coding regions. **B.** Respective FPKM values and confidence intervals for B6 and SJL female transcripts are shown (*q< 0.02). Q-values are false discovery rate-adjusted p-values.

Table 1

Top candidate modifier genes in Moe1.

Interval	Priority[†]	Gene Symbol	Gene Name
B	*	*Cltc*	Clathrin
	*	*Dynll2*	Dynein Light Chain 2
D-C	***	*Hlf*	Hepatic Leukemia Factor
C	***	*Cacna1g*	Voltage-Dep. Ca^{2+} Channel, T type, α1G
	***	*Cacnb1*	Voltage-Dep. Ca^{2+} Channel, β1
	**	*Slc25a39*	Solute Carrier Family 25, Member 39
	*	*Pnpo*	Pyridoxine 5′-Phosphate Oxidase
	*	*Hap1*	Huntingtin-Associated Protein 1

[†]Priority rating is based on biological function and prior association with neuronal hyperexcitability phenotypes.

***
highest priority;

**
moderate priority;

*
low priority.