IDENTIFICATION OF EPILEPSY MODIFIER GENES IN A MOUSE MODEL

By

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To my parents, for their endless support

and

To my husband, who fulfills my life, one giggle at a time

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure Page

LIST OF ABBREVIATIONS

General Abbreviations

α subunit	The major pore forming subunit of a channel
β subunit	The auxiliary subunit of a channel
129	129/SvJ Strain
ANOVA	Analysis of variance test
B6	C57BL/6J Strain
BAC	Bacterial artificial chromosome
BFNIS	Benign familial neonatal-infantile seizures
bp	Base pair
Ca^{2+}	Calcium
D1-D4	Domain 1-4, homologous domains in sodium channel α subunit topology
DBA	DBA/2J
ECoG	Electrocorticography
EEG	Electroencephalography
ENU	Ethylnitrosourea
F1	Outcross between two different inbred strains
FPKM	Fragments per kilobase of exon per million fragments mapped
GABA	Gamma-Aminobutyric acid
GEFS+	Genetic epilepsy with febrile seizure plus
GTCS	Generalized tonic-clonic seizure
HPLC	High performance liquid chromatography

IGE	Idiopathic generalized epilepsy
ISC	Interval-specific congenic
K+	Potassium
lncRNA	Long non-coding RNA
LOD	Logarithm of the odds
LVA	Low voltage activated calcium current
Mb	Megabase
MIT	Microsatellite markers
Moe1	Modifier of epilepsy 1, chromosome 11
Moe2	Modifier of epilepsy 2, chromosome 19
n	Number
ncRNA	Non-coding RNA
NS	Non-synonymous
P	Postnatal Day
PAR bZIP	Proline and acidic amino acid-rich basic leucine zipper
PDXK	Pyridoxal kinase
PLP	Pyridoxal 5’ phosphate
PLSD	Fisher’s protected least significant difference *post hoc* test
QTL	Quantitative trait locus
RIN	RNA Integrity number
S1-S6	Transmembrane segment 1-6
SJL	SJL/J Strain
SNPs	Single nucleotide polymorphisms

SUDEP	Sudden unexplained death in epilepsy
SWDs	Spike-wave discharges
TGF-β	Transforming growth factor β
TSS	Transcription start site
VCF	Variant call format

Common Gene Abbreviations

CACNA1G, Cacna1g	Human, Mouse: Calcium channel, voltage-dependent, T type, α 1G subunit
CACNB1, Cacnb1	Human, Mouse: Calcium channel, voltage-dependent, β 1 subunit
HLF, Hlf	Human, Mouse: Hepatic leukemia factor
KCNQ2, Kcnq2	Human, Mouse: Potassium channel, voltage-gated, KQT-like subfamily, member 2
KCNV2, Kcnv2	Human, Mouse: Potassium channel, subfamily 5, member 2
SCN1A, Scn1a	Human, Mouse: Sodium channel, voltage-gated, type 1, α subunit
SCN1B, Scn1a	Human, Mouse: Sodium Channel, voltage-gated, type 1, β subunit
SCN2A, Scn2a	Human, Mouse: Sodium channel, voltage-gated, type 2, α subunit
SCN3A, Scn3a	Human, Mouse: Sodium channel, voltage-gated, type 3, α subunit
SCN8A, Scn8a	Human, Mouse: Sodium channel, voltage-gated, Type 8, α subunit
SCN9A, Scn9a	Human, Mouse: Sodium channel, voltage-gated, type 9, α subunit

Mouse Alleles

1G5	*Cacna1g* Transgenic mouse
1G5;Q54	Double mutant 1G5 and *Scn2a*^{Q54}

ISC-A	B6-derived chromosome 11 segment 81-104 Mb on congenic SJL strain
ISC-B	B6-derived chromosome 11 segment 81-93 Mb on congenic SJL strain
ISC-C	B6-derived chromosome 11 segment 93-104 Mb on congenic SJL strain
ISC-D	B6-derived chromosome 11 segment 89-104 Mb on congenic SJL strain
Q54	*Scn2a*^{Q54}

CHAPTER I

INTRODUCTION

Epilepsy

Epilepsy is a common neurological disease defined as experiencing two or more unprovoked seizures separated by at least 24 hours (1, 2). While seizures differ in cause, severity and regions of brain involvement, all result from abnormal excessive or synchronous neuronal activity (3). Epilepsy is considered a spectrum disorder with more than 25 associated epilepsy syndromes, ranging from the severe, debilitating Dravet and Lennox-Gastaut syndromes to mild and treatable conditions, such as GEFS+ (Genetic Epilepsy with Febrile Seizure Plus) and absence epilepsies (4-6).

Epilepsy currently affects approximately 3 million Americans of all ages and 1% of the worldwide population (7). Two-thirds of patients diagnosed with epilepsy have no known cause for their disease, however recent evidence suggests most result from complex genetic interactions (8, 9). Roughly 20 years of research has resulted in major advancements in identifying genes that contribute to the monogenic epilepsies. The genes identified are components of neuronal signaling, including nicotinic acetylcholine and GABA receptors, chloride channels and voltage-gated potassium and sodium channels (10). Identification of these genes provides clues about the etiology of common epilepsy syndromes with more complex inheritance.

Voltage-Gated Sodium Channels

Voltage-gated sodium channels are responsible for the initiation and propagation of action potentials and are vital regulators of neuronal excitability. Voltage-gated sodium channel brain complexes were identified as a single α subunit associated with auxiliary β subunits (11). The α structure contains four homologous domains (D1-D4), each consisting of six α–helical transmembrane segments (S1-S6) (12). S4 of each domain is positively charged and contains the voltage sensors of the complex, which initiate channel activation (Figure 1.1) (13-19). Furthermore, there is evidence of S4/D4 having a unique role in inactivation (12, 16, 20). The β subunits (β1-β4) are single transmembrane segments that modulate voltage dependence, kinetics and localization of the α subunits (21, 22). The α subunits primarily expressed in the brain are encoded by *SCN1A*, *SCN2A*, *SCN3A* and *SCN8A* (12, 23-25). *SCN1A* and *SCN3A* channels are located mainly in neuronal cell bodies, *SCN2A* channels are localized to dendrites, unmyelinated or pre-myelinated axons and *SCN8A* channels are found in dendrites and the nodes of Ranvier (26-30).

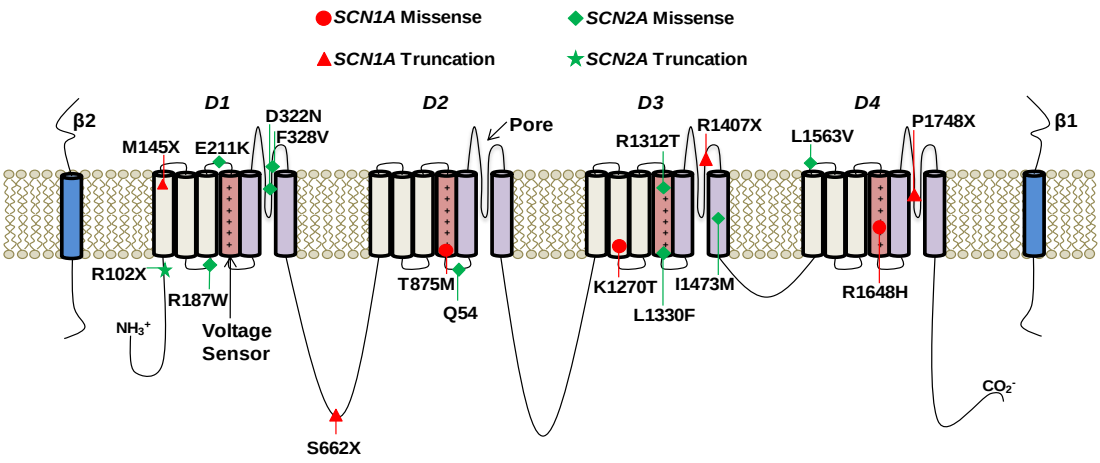

Figure 1.1 Voltage-Gated Sodium Channel Structure
Missense and truncation mutations of *SCN1A* and *SCN2A* described in the introduction are indicated.

Voltage-Gated Sodium Channels and Epilepsy

Currently, nearly 900 mutations in *SCN1A* have been reported in patients with various types of epilepsy, making it the most common cause of monogenic epilepsy (31). Mutations in voltage-gated sodium channels are responsible for several types of human epilepsy, including GEFS+ and Dravet Syndrome, formerly known as Severe Myoclonic Epilepsy of Infancy (10). GEFS+ is a benign, childhood-onset syndrome with autosomal dominant inheritance (OMIM 604233). This familial syndrome is characterized by febrile seizures that persist beyond six years of age and afebrile, generalized seizure types later in life (32, 33). In 1998, the GEFS+ mutation *SCN1B*^{C121W} was identified in the β1 subunit gene (34). The effect of this mutation was impaired modulation of the sodium channel α subunit (35). In 1999, linkage analysis of two large families identified a second GEFS+ locus localized to chromosome 2 (36, 37). The nonrecombinant interval contained a sodium channel gene cluster that includes *SCN1A*, *SCN2A*, *SCN3A* and *SCN9A*. It was found that affected individuals from one family were heterozygous for *SCN1A*^{R1648H} and affected members from another family were heterozygous for *SCN1A*^{T875M} (38). When the *SCN1A*^{T875M} and *SCN1A*^{R1648H} mutations were heterologously expressed with β1 and β2 subunits, both GEFS+ mutations exhibited noninactivating inward sodium currents (39). Additionally, single channel analysis of *SCN1A*^{R1648H} activity revealed mutants had a higher probability of late channel openings (39). The data suggest that these GEFS+ variants result in gain-of-function mutations caused by defects in sodium channel inactivation, thereby enhancing sodium current. Persistent sodium influx is hypothesized to extend neuronal depolarization, initiate hyperexcitability and increase seizure susceptibility (39).

Dravet syndrome is the phenotype most frequently associated with mutations of *SCN1A* (OMIM 607208) (40). Over 80% of reported *SCN1A* mutations have been identified in Dravet syndrome or its variant, severe myoclonic epilepsy, borderline (SMEB) (31, 40). Approximately 90% of *SCN1A* mutations found in Dravet patients arose *de novo* and nearly half are nonsense, frameshift or splice site mutations resulting in protein truncation (10, 41, 42). This syndrome is characterized by infant-onset generalized clonic, generalized tonic-clonic or hemiclonic seizures, often precipitated by fever. Although development is normal prior to disease onset, progression is often coupled to a severe decline of psychomotor and cognitive development (43, 44). Dravet patients develop other types of seizures, including absence and partial seizures and respond poorly to anti-epileptic drugs.

Recently, seven *SCN1A* variants were identified by exome sequencing of patients with infantile spasms or Lennox-Gastaut (45). These epileptic encephalopathies, like Dravet syndrome, are severe epilepsy disorders characterized by early seizure onset with cognitive and behavioral delays due to regular epileptic activity (45).

A smaller number of mutations have been reported in the other neuronal sodium channel genes *SCN2A*, *SCN3A* and *SCN8A*. *SCN2A* is a paralogous sodium channel gene closely linked to *SCN1A* on chromosome 2. Several missense mutations in *SCN2A* have been detected in patients with Benign Familial Neonatal-Infantile Seizures (BFNIS), GEFS+, Dravet and Ohtahara syndromes (46, 47). Interestingly, recent studies have attributed several *SCN2A* mutations to intellectual disability and autism (48, 49). This is of particular significance as estimates predict epilepsy affects 7-46% of autism spectrum

patients (50-52). Furthermore, epilepsy occurs more often in autism patients who also have an intellectual disability (53).

BFNIS is characterized by the onset of afebrile generalized seizures typically between 2 days and 3.5 months of life, which spontaneously remit by age one (54). Twelve *SCN2A* missense mutations have been found in BFNIS patients (55-60). These BFNIS *SCN2A* mutants exhibit a wide range of functional abnormalities hypothesized to contribute to seizure generation (58-62). Two mutations, $SCN2A^{R187W}$ and $SCN2A^{R1918H}$, have been identified in GEFS+ patients (63, 64). Electrophysiological recordings illustrate abnormalities that may be responsible for the hyperexcitability leading to seizures at the neuronal level (64). Seven missense mutations and the nonsense mutation $SCN2A^{R102X}$ were all identified in Dravet patients (65-69). Several *SCN2A* Dravet mutations have been expressed in heterologous systems and displayed abnormalities suggestive of epileptiform activities (64, 67, 70).

The $SCN2A^{A263V}$ missense mutation was identified in a set of monozygotic twins with Ohtahara syndrome (47). Ohtahara syndrome is classified as an early onset epileptic encephalopathy, characterized by developmental impairment and devastating seizures starting at birth (71). The Ohtahara diagnosis was based on neonatal seizures and a distinct EEG pattern. Additionally, both twins had brain structure abnormalities, characteristic of a progressive neurodegenerative disease (47). Furthermore, the $SCN2A^{R853Q}$ missense mutation was identified in two unrelated patients, diagnosed with either infantile spasms or Lennox-Gastaut, two epileptic encephalopathies (45).

Five mutations of *SCN3A* have been identified in childhood epilepsy. The missense mutation $SCN3A^{K345Q}$ was found in an individual with partial epilepsy (72).

Functional analysis of *SCN3A*^{K345Q} revealed altered sodium currents providing a pathophysiological basis for the epilepsy linked mutation (73). Our laboratory identified four additional *SCN3A* mutations in a cohort of pediatric patients with cryptogenic focal epilepsy (74). *In vitro* analysis of all four mutations demonstrated significant functional defects including increased levels of persistent current and increased ramp current (74). Alterations in ramp current and persistent current have both previously been indicated in epilepsy related mutations (39, 72, 75-78).

Advances in whole genome sequencing allowed for recent identification of *SCN8A* mutations involved in the severe epileptic encephalopathies. The *SCN8A*^{N1768B} missense mutation resulted in early-onset seizures, autism, intellectual disability, ataxia and sudden unexplained death in epilepsy (SUDEP) (79). The functional consequences of the mutation were evaluated *in vitro*, revealing a remarkable increase in sodium current, incomplete channel inactivation, increased spontaneous firing and firing frequency (79). Two additional missense mutations, *SCN8A*^{G214D} and *SCN8A*^{L875Q}, were identified in patients diagnosed with infantile spasms or Lennox-Gastaut. These *SCN8A* mutations are predicted to increase neuronal excitability, which has previously been implicated in seizure development.

Several microdeletions and duplications of the voltage gated sodium channel gene cluster (2q24) have been identified. Chromosomal deletions of 2q24 have been described in patients who suffer from epilepsy (80-94). A broad disparity in seizure characteristics and diagnosis has been detailed in 2q24 deletions, ranging from infantile spasms, minor convulsions and sporadic myoclonic jerks to generalized tonic-clonic seizures, status epilepticus and Dravet syndrome (83). Five cases of 2q24 duplications have been

implicated in epilepsy. These duplications all resulted in early-onset infantile seizures and global developmental delays (95-99).

Variable Expressivity of Sodium Channel Mutations in Epilepsy

A common feature of inherited epilepsy due to a sodium channel mutation is that family members who carry the same mutation often display a difference in the clinical severity of the disease. This is seen in both GEFS+ and Dravet syndrome. In a GEFS+ family carrying the *SCN1A*^{T875M} mutation, half of the affected family members had only febrile seizures, while the other mutation carriers had more severe types of seizures as adults, including generalized tonic-clonic, absence, atonic and hemicorporeal clonic seizures (37, 38). In another GEFS+ family with the *SCN1A*^{K1270T} mutation, eleven family members had only febrile seizures plus and five family members had evidence of temporal lobe epilepsy (100).

The *SCN2A*^{A263V} mutation implicated in monozygotic twins with Ohtahara syndrome was previously identified in a patient with an epileptic encephalopathy resembling BFNIS (59). While the BFNIS patient had neonatal onset seizures and late onset episodic symptoms, the dramatic characteristics identified in the Ohtahara patients were not observed (47, 59).

In rare cases, Dravet patients have inherited an *SCN1A* mutation from a mildly affected parent (101-103). Gennaro and colleagues described a family in which two siblings with Dravet syndrome inherited the *SCN1A*^{P1748fsX1779} mutation from their mother, who had only a single febrile seizure in childhood (102). Two *SCN1A* truncation mutations have been identified that did not result in Dravet syndrome (104). Instead,

SCN1A^{S662X} and *SCN1A*^{M145fsx148} produced GEFS+ and focal seizures, much milder forms of epilepsy not commonly associated with *SCN1A* truncations (104).

Variable expressivity suggests that other factors in addition to a primary mutation influence the clinical manifestation of epilepsy. This could include stochastic events during development, accumulation of somatic mutations during the lifetime of an individual or environmental insults such as trauma (105). Variation in inheritance of other genetic risk alleles in different family members may also modify the clinical severity of epilepsy.

Epilepsy Models With Sodium Channel Mutations

Several mouse models with seizure-related phenotypes recapitulating GEFS+ and Dravet syndrome have been generated by introducing mutations in voltage-gated sodium channel α subunits. A GEFS+ model was developed by knocking-in the *SCN1A*^{R1648H} mutation into the orthologous mouse gene (106). The *Scn1a*$^{tm1.1Aesc}$ mouse allele is referred to as *Scn1a*^{R1648H}. To study Dravet syndrome, we and others have developed targeted null alleles of *Scn1a* (107, 108). The mouse model *Scn1a*^{tm1Kzy}, a knock-in of the *SCN1A*^{R1407X} mutation, was generated to examine the effects of truncated *Scn1a* (109).

SCN1A^{R1648H} was the first human GEFS+ mutation studied *in vivo*. *Scn1a*$^{R1648H/R1648H}$ homozygous mice experienced premature lethality by postnatal day 26 (P26) (106). Behavioral observations revealed both heterozygous and homozygous mutant animals exhibited spontaneous, generalized seizures that were confirmed by electrocorticography (ECoG) recordings (106). Heterozygous mutant mice were more susceptible to seizure induction by the chemiconvulsant flurothyl and therefore had

reduced times to seizure onset (106). Flurothyl-induced seizure thresholds were restored to wildtype levels by administration of the anticonvulsant valproic acid, commonly used to treat GEFS+ patients, validating the knock-in as a model for GEFS+ (106). Electrophysiological analysis revealed cortical GABAergic bipolar interneurons from heterozygous *Scn1a*$^{R1648H/+}$ and homozygous *Scn1a*$^{R1648H/R1648H}$ mice displayed reduced total sodium current amplitudes, increased use-dependence and slower recovery from inactivation (106). Additionally, homozygous animals had a significant reduction in action potential firing in these interneurons (106). It is hypothesized that reduced excitability of GABAergic interneurons is a key contributor to seizure generation in the *Scn1a*$^{R1648H/+}$ GEFS+ model (106).

A Dravet mouse model, *Scn1a*^{tm1Wac}, was generated by disruption of the *Scn1a* gene. *Scn1a*$^{KO/KO}$ homozygous null mice exhibited spontaneous seizures and ataxia by P9, with premature lethality by P15 (110). *Scn1a*$^{KO/+}$ heterozygous mice displayed frequent, spontaneous seizures that were confirmed by ECoG recordings (110). *Scn1a*$^{KO/+}$ heterozygotes experienced sporadic death beginning at P21, with 40% lethality by the 15th week of life (110). Electrophysiological analysis of isolated neurons revealed a significant reduction of sodium current levels in hippocampal GABAergic interneurons from *Scn1a*$^{KO/+}$ heterozygous and *Scn1a*$^{KO/KO}$ homozygous mice (110). GABAergic interneurons also displayed significant decreases in action potential firing, frequency and amplitude in both *Scn1a*$^{KO/+}$ heterozygous and *Scn1a*$^{KO/KO}$ homozygous mice (110). These abnormalities are indicative of reduced GABAergic transmission, a major contributor to neuronal hyperexcitability and seizure generation. In addition to the seizure phenotype, *Scn1a*$^{KO/+}$ heterozygous mice display autism-spectrum features, including

hyperactivity, stereotyped behaviors, social interaction deficits and impaired context-dependent spatial memory (111). SUDEP was also shown to occur in *Scn1a*$^{KO/+}$ heterozygous mice (112). SUDEP in *Scn1a*$^{KO/+}$ mice is hypothesized to occur from seizure-induced parasympathetic hyperactivity resulting in lethal bradycardia and electrical dysfunction in the heart (112).

An additional knock-in model of Dravet was generated carrying a premature stop codon recapitulating the human *SCN1A*^{R1407X} mutation identified in three unrelated patients (101, 109, 113, 114). At P12, homozygous *Scn1a*$^{R1407X/R1407X}$ mice exhibited tonic-clonic seizures confirmed by ECoG and experienced premature lethality by P21 (109). Heterozygous *Scn1a*$^{R1407X/+}$ mice developed seizures at P21, with a 40% mortality rate by three months of age, similar to the heterozygous *Scn1a*$^{KO/+}$ null allele (109).

Our laboratory has developed the mouse model Tg(Eno2-Scn2a1*)Q54Mm with a missense mutation of *Scn2a*, designated *Scn2a*^{Q54} (Q54), which resembles an epilepsy phenotype of human patients. The transgenic Q54 mouse has a gain-of-function mutation [GAL(879-881)QQQ], located in the S4-S5 intracellular linker in D2. Q54 mice have a progressive epilepsy phenotype which begins with brief, focal motor seizures (115). As they age, Q54 mice exhibit more frequent focal seizures, along with secondarily generalized seizures and have a reduced lifespan (115). Recordings of isolated excitatory hippocampal pyramidal neurons from Q54 mice revealed increased persistent sodium current which is hypothesized to contribute to seizure generation by increasing neuronal hyperexcitability (115). Functional studies of several human GEFS+ mutations in heterologous expression systems have also shown increased persistent current of similar magnitude (39, 77, 116). Hippocampal slice recordings from Q54 mice demonstrated

network hyperexcitability during recording of spontaneous and evoked activity, supporting the hypothesis that increased persistent current leads to neuronal hyperexcitability (117).

Genetic Background Influences Epilepsy Models

A common feature of seizure mouse models, including sodium channel mutants, is that seizure frequency and animal survival varies significantly depending on the genetic background of the mouse. This supports the idea of genetic modifiers in epilepsy. Consequently, these 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 crossing the mutation onto different background strains. Modifier loci have been mapped for several diseases in human and mouse, including cystic fibrosis, long QT syndrome, cancer, retinal, cochlear and motor neuron degeneration, aganglionosis, otocephaly, tremor and dystonia (118-126). Modifier genes have been identified for several of these loci (120, 126-132).

Early studies revealed that there were considerable differences in seizure susceptibility dependent on genetic strain background (133, 134). Ferraro and colleagues demonstrated that genetic factors in C57BL/6J (B6) and DBA/2J (DBA) influence seizure susceptibility, with the B6 strain being far more seizure resistant than the DBA strain to a variety of seizure induction methods (135-140). Genetic mapping approaches identified *Kcnj10*, an inward rectifying potassium channel, as a candidate gene for seizure susceptibility in these two strains (141, 142).

Scn1a$^{KO/+}$ heterozygotes on the B6 strain have an 80% lethality rate by 13 weeks of age and frequent, spontaneous seizure activity (107). When *Scn1a*$^{KO/+}$ was bred to and maintained on the 129/SvJ (129) strain, only 10% of heterozygotes died by 15 weeks and seizure activity was not observed (107). Loss of *Scn1a* is more severe on the B6 strain, suggesting the 129 strain contributes modifier genes that improve the epilepsy phenotype. Also, the *Scn1a*^{R1407X} mutation was found to be far less penetrate on the 129 strain (109).

Q54 mice on the B6 strain exhibit a low incidence of seizures, with less than 20% having seizures at three months of age and a 75% survival rate to six months of age (143). When B6.Q54 mice are crossed to the SJL/J (SJL) strain, the resulting F1 generation display a high incidence of seizures (>80%) with early onset and only 25% survival to six months of age (143). This observation indicates the SJL strain contributes dominant modifiers which affect the severity of the epilepsy phenotype (143). Genetic mapping was performed which identified two modifier loci responsible for the strain difference: *Moe1* (Modifier of Epilepsy 1) and *Moe2* (143). The voltage-gated potassium channel *Kcnv2* was identified as the modifier gene for *Moe2* (144, 145). Identification and verification of modifier genes in *Moe1* is discussed in Chapters II and III.

Genetic Modifiers of Epilepsy

The idea of genetic modifiers influencing clinical severity is becoming increasingly important for understanding the pathophysiology of inherited disease. Several genes have already been identified in mouse models which alter seizure phenotype and verified in human epilepsy patients (145, 146)

Scn8a has been identified as a modifier of Dravet syndrome. The *Scn8a*$^{med\text{-}jo}$ mouse model contains a missense mutation that results in neuromuscular abnormalities, but has increased thresholds to seizure induction (147-152). When *Scn8a*$^{med\text{-}jo}$ heterozygotes were crossed with *Scn1a*$^{KO/+}$ heterozygotes to generate double heterozygous mutants, the *Scn8a*$^{med\text{-}jo}$ allele was able to rescue reduced seizure thresholds of *Scn1a*$^{KO/+}$ mice and improve their premature lethality (148). These findings suggest *Scn8a* can improve the severity of the *Scn1a* seizure phenotype.

The calcium channel mutant *Cacna1a*$^{tg/tg}$ recapitulates absence epilepsy and was identified as a modifier of the temporal lobe epilepsy model *Kcna1*. *Kcna1* mutants lack the Kv1.1 α-subunit of the Shaker-type potassium (K^+) channels and therefore the loss of K+ channel regulation of action potential firing. *Kcna1*$^{KO/KO}$ null mutants experienced premature lethality with a 26% survival rate to ten weeks of age and frequent, severe, tonic-clonic seizures (153). When *Cacna1a*$^{tg/tg}$ is crossed to *Kcna1*$^{KO/KO}$ nulls to generate double homozygous mutants, 87% of animals survived to ten weeks of age (153). Additionally, double homozygous mutants lacked absence seizures and had a 60% and 80% reduction in tonic-clonic frequency and length of seizure, respectively (153). The modifier effect is hypothesized to be the reduced neurotransmission of *Cacna1a*$^{tg/tg}$ diminishing *Kcna1*$^{KO/KO}$ hyperexcitability.

Recent work has suggested human variants in *CACNA1A* and *SCN9A* modify the Dravet syndrome phenotype. Patients with mutations in both *CACNA1A* and *SCN1A* experienced higher rates of absence seizures, earlier seizure onset and significantly more prolonged seizures compared to Dravet patients with only a *SCN1A* mutation (146). The *SCN9A*^{N641Y} mutation was identified in a large family with 21 individuals experiencing

febrile seizure before six years of age (154, 155). Ten of the affected family members continued with subsequent afebrile seizures (154, 155). Due to the expansive phenotype seen in *SCN9A*^{N641Y} patients, it was hypothesized that *SCN9A* may act as a genetic modifier of epilepsy. Singh *et al*. identified *SCN9A* mutations in seven Dravet syndrome patients. Six of these patients were also positive for mild *SCN1A* missense mutations not usually associated with Dravet (155). Furthermore, five *SCN9A* variants were identified in 18 Dravet syndrome patients negative for *SCN1A* mutations (156). The same study also identified seven additional *SCN9A* variants in *SCN1A* positive Dravet patients (156). The results suggest that variants in *SCN9A* alone or in combination with *SCN1A* missense mutations, result in Dravet syndrome (155, 156).

We demonstrated the voltage-gated potassium and sodium channel mutants *Kcnq2*$^{V182M/+}$ and *Scn1a*$^{R1648H/+}$ were modifiers of the Q54 seizure model (157, 158). *Kcnq2*$^{V182M/+}$ heterozygous animals have a reduced threshold to seizure induction but no spontaneous seizure activity (159). When *Kcnq2*$^{V182M/+}$ heterozygotes were combined with Q54, double mutants experienced early-onset, tonic-clonic seizures and juvenile lethality by three weeks of age, unlike the single mutant littermates (158). When *Scn1a*$^{R1648H/+}$ heterozygotes were combined with Q54, double heterozygous mutants experienced 100% mortality by P24 and frequent, spontaneous tonic-clonic seizures (157). These experiments are detailed in Chapter IV. These modifier effects demonstrate that neuronal excitability is influenced by the net activity of ion channels (157).

Supporting the role for human genetic modifiers of epilepsy, mutations have been identified in *CACNA1A*, *KCNJ10*, *KCNQ2* and *KCNV2* in patients with absence, generalized, BFNIS and partial epilepsies, respectively (160-165).

Conclusions and Specific Aims

As described throughout the introduction, epilepsy is a common neurological disorder with a wide spectrum of phenotypic severity. Among monogenic epilepsies, like Dravet syndrome and GEFS+, affected family members who possess the same mutation can have dramatic differences in the clinical presentation of their disease. This suggests that other factors, including genetic, must modify the primary mutation, resulting in a more or less severe phenotype.

Genetic epilepsies with complex inheritance likely account for a considerable amount of epilepsies with unknown origin (166). In complex inheritance, each individual gene may have only a small effect on clinical severity of epilepsy, but in combination with risk alleles in other genes, the additive effects can be devastating.

Mutations in voltage-gated sodium channels are major contributors to genetic epilepsies, with 80% of Dravet syndrome and 10% of GEFS+ patients having mutations in *SCN1A* (167). Generation of mouse models recapitulating voltage-gated sodium and other neuronal ion channel mutations have allowed for more accurate models of Dravet syndrome, GEFS+, absence and temporal lobe epilepsies. The *in vivo* models are not only beneficial to assess behavioral consequences of ion channel mutations, but are also used to study the molecular mechanisms altered by mutations, therefore expanding our understanding of epilepsy. An increasing amount of evidence from these models has shown that seizure phenotypes resulting from gene mutations are modified by the genetic strain background.

The Q54 transgenic mouse model has a gain-of-function mutation that results in persistent sodium current and epilepsy. Q54 mice on the B6 strain exhibit a low incidence

of seizures and a relatively normal lifespan (143). When crossed to the SJL strain, the resulting F1.Q54 offspring display a high seizure frequency with early onset and significantly reduced survival (143). Genetic mapping identified two modifier loci responsible for the strain difference in Q54 mice: *Moe1* and *Moe2* (143, 145).

I proposed to identify and evaluate candidate genes that alter seizure susceptibility at the *Moe1* locus through the following specific aims:

Specific Aim 1. ***To fine map Moe1 on chromosome 11 to ≤ 10 Mb using interval-specific congenics and perform RNA-Seq to identify candidate genes.***

Specific Aim 2. ***To test Moe1 candidate modifier genes by transgenic transfer of the modified phenotype.***

In Specific Aim 1, I utilized interval-specific congenic lines to fine-map *Moe1* and narrow the modifier interval. We then used a next-generation sequencing technique, RNA-Seq, to evaluate differences in gene and transcript expression, as well as coding sequence, between B6 and SJL strains. Fine mapping and RNA-Seq suggested several candidate modifier genes, including *Cacna1g* and *Hlf,* for *Moe1*. These results are described in detail in Chapter II, modified from *Hawkins & Kearney, 2012*. I hypothesized candidate genes identified by fine mapping and RNA-Seq contributed to the variable phenotype of Q54.

In Specific Aim 2, I tested the modifier potential of *Cacna1g* and *Hlf* by systematically evaluating the candidate modifier genes through generation of *Cacna1g* BAC transgenic and *Hlf* targeted knockout animal models. I tested their ability to enhance or reduce seizure severity of the Q54 phenotype by generating double mutant

animals. Descriptions of these experiments and validation of *Cacna1g* and *Hlf* as candidate modifier genes of the Q54 phenotype are detailed in Chapter III.

I also proposed Specific Aim 3, which focused on taking a functional candidate gene approach to evaluate known epilepsy mutations on the *Scn1a*$^{R1648H/+}$ phenotype.

Specific Aim 3. To test the Q54 and Kcnq2$^{V182M/+}$ epilepsy models as functional candidate modifiers of the GEFS+ knock-in model Scn1a$^{R1648H/+}$.

To assess the modifier potential of Q54 and *Kcnq2*$^{V182M/+}$ mutations on the *Scn1a*$^{R1648H/+}$ phenotype, we generated double mutant mice. I then utilized ECoG to monitor seizure activity in our mutants. I hypothesized that by combining reduced GABAergic transmission from *Scn1a*$^{R1648H/+}$ with reduced seizure thresholds from the *Kcnq2*$^{V182M/+}$ mutation or hyperexcitability from Q54, a severe seizure phenotype would result in each cross. These results are described in detail in Chapter IV, modified from *Hawkins et al., 2011*.

Identification of modifier genes that improve or exacerbate epilepsy may increase the understanding of the molecular events of epileptogenesis, advance molecular diagnostic capabilities and identify novel therapeutic targets for the improved treatment of human patients

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CHAPTER II

CONFIRMATION OF AN EPILEPSY MODIFIER LOCUS ON MOUSE CHROMOSOME 11 AND CANDIDATE GENE ANALYSIS BY RNA-SEQ*

Introduction

Mutations in voltage-gated sodium channels are responsible for several types of human epilepsy (1). Mutations in *SCN1A* were first identified in patients with Genetic Epilepsy with Febrile Seizure Plus (GEFS+) (2). Subsequently, *SCN1A* mutations were identified in patients with Dravet, a severe infant-onset epileptic encephalopathy (3). To date, nearly 900 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+, Dravet and Ohtahara syndromes. (4). Affected family members with the same sodium channel mutation often display variability in the clinical severity of their 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 complex genetics, for example genetic modifiers, contribute to the variable phenotype observed in seizure models. 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

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can be identified systematically by evaluating the seizure phenotype on different background strains. Several strain-dependent seizure susceptibility loci and genes have already been identified (5-16).

The transgenic mouse model *Scn2a*^{Q54} (Q54) has an epilepsy phenotype due to a mutation in *Scn2a* that slows channel inactivation and results in persistent sodium current (17). Severity of the epilepsy phenotype in 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 11 and *Moe2* on chromosome 19, that influence severity of the Q54 phenotype (18). Fine mapping of *Moe2* identified *Kcnv2* as a strong candidate gene (19). Transgenic transfer of the modified phenotype *in vivo* supported the identification of *Kcnv2* as a modifier gene in mice (20). Discovery of two novel human *KCNV2* mutations in pediatric epilepsy patients suggested that *KCNV2* may also contribute to human epilepsy susceptibility (20).

In this study, we generated interval-specific congenic (ISC) lines to confirm and fine map the *Moe1* locus on chromosome 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 single-nucleotide polymorphisms (SNPs) between sex and strain. We further evaluated and prioritized candidate modifier genes that may contribute to strain-dependent differences in seizure susceptibility in Q54 mice.

Materials and Methods

Mice

Q54 transgenic mice [Tg(Eno2-Scn2a1*)Q54Mm] were generated as previously described (17). The Q54 line congenic on the B6 strain (B6.Q54) was established as described and is maintained by continued backcrossing of B6.Q54 hemizygous males to B6 females (Jackson Labs stock #000664) (18). 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 differing B6-derived chromosome 11 segments within *Moe1* on a congenic SJL background. B6 males were crossed with SJL females to create F1 progeny. Offspring from each generation 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 (Illumina, San Diego, CA, USA) (21). 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 chromosome 11 microsatellite (MIT) 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, Valencia, CA, USA). The Q54 transgene was genotyped as previously described (18). ISC lines were genotyped for chromosome 11 MIT markers by polymerase chain reaction and analyzed on 2% agarose or 7% non-denaturing polyacrylamide gels.

Phenotyping

Chromosome 11 ISC females were crossed with hemizygous B6.Q54 males to generate (B6.Q54 × ISC.SJL).F1 offspring (F1.Q54) carrying heterozygous or homozygous B6 alleles in *Moe1*. We used a similar phenotyping paradigm used for low-resolution mapping of the *Moe1* locus (18). Briefly, F1.Q54 offspring underwent 30 minute video-taped observations at three and six weeks of age. All observation sessions occurred between 1:00 PM and 4:00 PM. On the basis of prior extensive video-electroencephalogram monitoring, spontaneous behavioral seizures were assessed offline by a blinded observer using Observer XT software (Noldus, Wageningen, Netherlands) (17). We counted the number of visible focal motor seizures with forelimb clonus and repetitive movements lasting one to five seconds (Racine seizure scale 3) (22). Seizure counts from the three and six week observations were combined to obtain a frequency of seizures in 60 minutes for each animal.

Statistical Analysis

Average seizure frequencies were compared between ISC lines and controls by analysis of variance (ANOVA) with Fisher's protected least significant difference (PLSD) *post hoc* test ($n \geq 9$ per group). Outlier analysis was performed and subjects with seizure frequencies two standard deviations above and below the mean at three or six weeks of age were removed. Mice with missing three or six week data due to outlier frequencies or death were not included in the study. Preliminary analysis indicated that males and females differed within the strains. Therefore, male and female data were analyzed separately. Survival was assessed until 12 weeks of age using Kaplan-Meier analysis with P-values reported from Logrank Mantel-Cox.

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, Carlsbad, CA, USA). RNA integrity was assessed on an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA) and all samples received RNA integrity numbers (RIN) of ≥8.20. For each group, total RNAs from four mice were combined to generate a pooled sample.

Sample Preparation for RNA-Seq

Starting with three 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, San Diego, CA, USA). Following second strand

cDNA synthesis, samples were prepared for sequencing on the Illumina HiSeq 2000 platform in the Vanderbilt Technologies for Advanced Genomics Core (http://vantage.vanderbilt.edu) according to standard methods (23). 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 (23). Subsequent analysis was performed using Tuxedo Tools on the open, web-based platform GALAXY (24-26). 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 (27-29). 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 Cufflink transcript data across sex and strain. Cuffdiff tested for statistically significant differences in total gene and transcript expression, splicing, transcription start sites (TSS) and promoter usage (30-32). Parameter settings for each analysis tool are detailed in Table 2.1.

Variant calls were performed by the Vanderbilt Computational Genomics Core using the Genome Analysis Toolkit for SNP discovery (http://www.broadinstitute.org/gsa/wiki/index.php/Main_Page). 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 groups with missing sample data. SNPs that were homozygous alternate from the B6 reference genome were retained.

Variant call format (VCF) files were uploaded to Ensembl's Variant Effect Predictor to reveal the functional consequences of identified variants (33). Non-synonymous amino acid substitutions were uploaded to sift (http://sift.jcvi.org/) to predict the potential impact on protein function (34).

Table 2.1 Parameter Settings for GALAXY RNA-Seq Analysis

RNA-Seq analysis was performed by GALAXY using the following parameters. RNA-Seq reads were aligned by Tophat to the mm9 genome by Bowtie. Cufflinks assembled transcripts, estimated abundance in FPKM and tested for differential expression and regulation. Cuffcompare combined Cufflinks transcript data across sex and strain. Cuffdiff tested for statistically significant differences in total gene and transcript expression, splicing, TSS and promoter usage.

Tool	Parameter	Setting
Tophat (V. 1.1.2)	Anchor length	8
	Max. number of mismatches in the anchor region of spliced alignment	0
	Min. intron length	70
	Max. intron length	500000
	Allow indel search	Yes
	Max insertion length	3
	Max deletion length	3
	Max. number of alignments allowed	20
	Min. intron length found during split-segment (default) search	50
	Max. intron length found during split-segment (default) search	500000
	Number of mismatches allowed in the initial read mapping	2
	Number of mismatches allowed in each segment alignment for reads mapped	2
	Min. length of read segments	25
	Use own junctions	No
	Use closure search	No
	Use coverage search	Yes
	Min. intron length that may be found during coverage search	50
	Max. intron length that may be found during coverage search	20000
	Use microexon search	No
Cufflinks (V. 0.0.5)	Max. intron length	300000
	Min. isoform fraction	0.1
	Pre MRNA fraction	0.15
	Perform quartile normalization	No
	Use reference annotation	Yes
	Perform bias correction	No
	Set parameters for paired-end reads	No
Cuff-compare (V. 0.0.5)	Use reference annotation	Yes
	Use sequence data	Yes
	Choose the source for the reference list	Local
Cuffdiff (V. 0.0.5)	Perform replicate analysis	No
	False discovery rate	0.05
	Min. alignment count	1000
	Perform quartile normalization	No
	Perform bias correction	No
	Set parameters for paired-end reads	No

Results

Interval-Specific Congenics

We previously mapped the Q54 modifier *Moe1* to mouse chromosome 11 with a peak at D11MIT289 (95 Mb) (18). 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. Each line contained varying B6-derived chromosome 11 segments within the *Moe1* 1 LOD support interval on a congenic SJL background. ISC lines are represented by their MIT marker location. The resulting ISC lines were as follows: 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) (Figure 2.1).

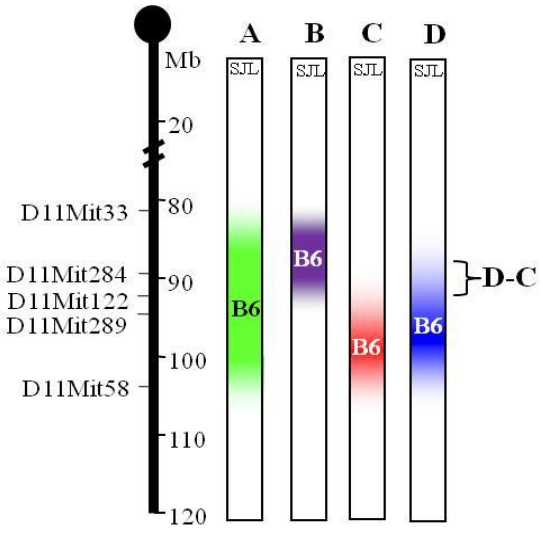

Figure 2.1 Fine Mapping of *Moe1* with ISC Lines
ISC lines were generated with varying B6-derived chromosome 11 segments (colors) on an SJL background (white). MIT markers used for ISC generation are identified along the chromosome. The original mapping for epilepsy susceptibility localized the *Moe1* peak to D11Mit289.

ISC Phenotyping

Chromosome 11 ISC females were crossed with B6.Q54 males to generate F1.Q54. Offspring were heterozygous or homozygous for B6 alleles in *Moe1* (Figure 2.2). Genotypes for the F1 generation 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.

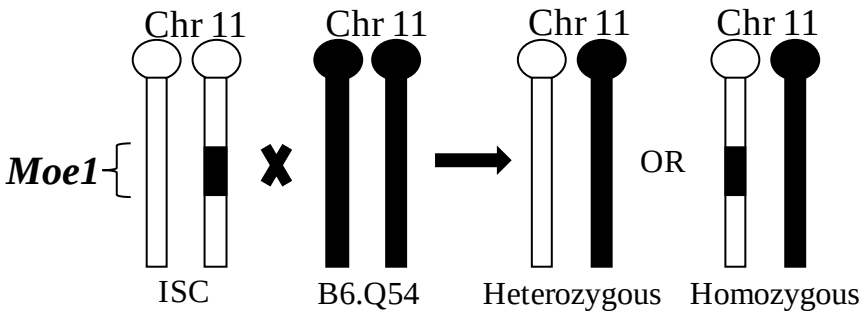

Figure 2.2 ISC Phenotyping Paradigm
ISC lines were crossed with B6.Q54, generating F1.Q54 offspring. F1.Q54 mice will be heterozygous or homozygous for B6 alleles in *Moe1*.

A significant difference in seizure frequency was identified between ISC males and controls by ANOVA ($F_{4,70} = 3.321$, $p < 0.015$). Male mice carrying homozygous B6 alleles in the ISC-D interval (89–104 Mb) exhibited more seizures than heterozygous control littermates ($p < 0.003$) (Figure 2.3). Interestingly, males with homozygous B6 alleles in the ISC-C interval (93-104 Mb) showed no difference compared to heterozygous control littermates ($p > 0.496$) (Figure 2.3). 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.

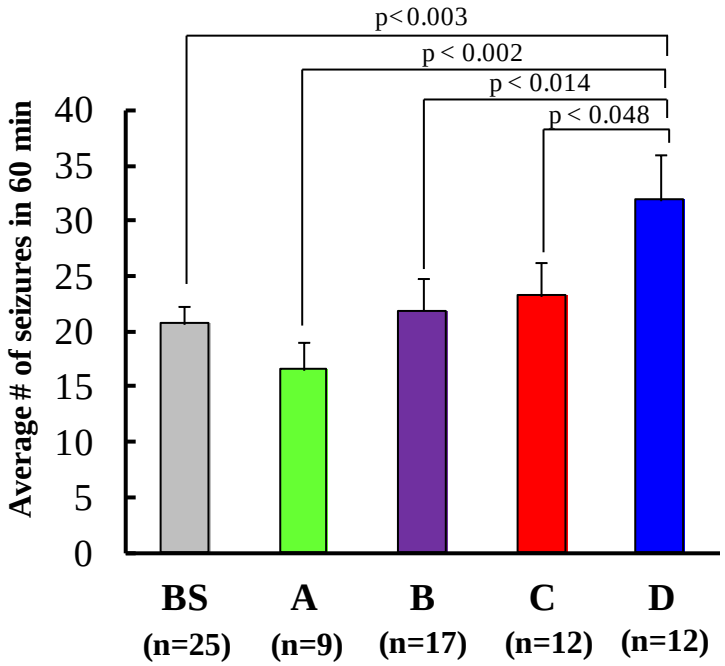

Figure 2.3 Seizure Frequency in ISC Males
Average number of seizures in 60 minutes for each genotype group is shown. Significant differences between genotype groups were determined by ANOVA: $F_{4,70}$= 3.321, p < 0.015 with significant p-values determined by Fishers PLSD *post hoc* test. Data are represented as mean ± SEM.

A significant difference in seizure frequency was identified between ISC females and controls by ANOVA ($F_{4,70}$ = 2.601, p < 0.043). Female mice carrying homozygous B6 alleles in the ISC-C interval (93–104 Mb) exhibited more seizures than heterozygous control littermates (p < 0.047) (Figure 2.4). 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 (p > 0.805). This suggests the male

modifier located 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.

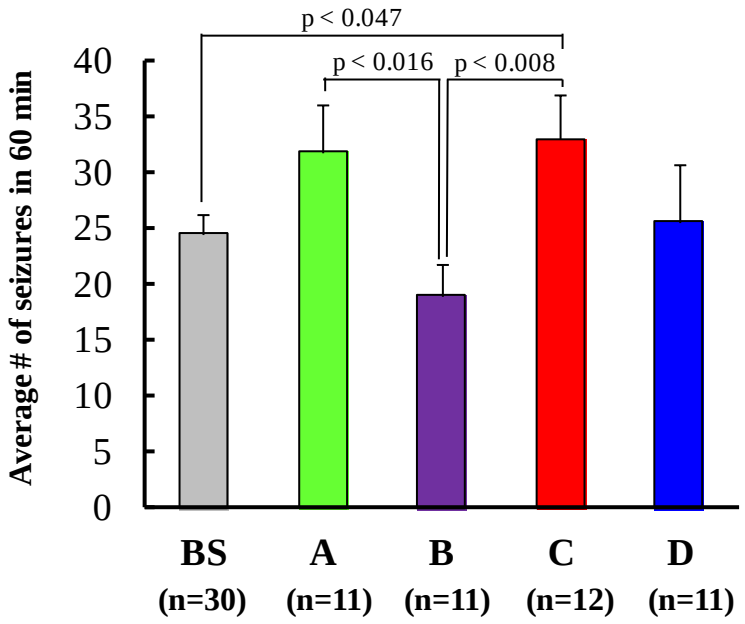

Figure 2.4 Seizure Frequency in ISC Females
Average number of seizures in 60 minutes for each genotype group is shown. Significant differences between genotype groups were determined by ANOVA: $F_{4,70}$= 2.601, p < 0.043 with significant p-values determined by Fishers PLSD *post hoc* test. Data are represented as mean ± SEM.

In both sexes, B6 homozygosity in the large ISC-A region (81–104 Mb) that spans the entire *Moe1* 1 LOD support interval did not result in significant differences in seizure frequency compared to heterozygous controls (Males: p > 0.312; Females: p > 0.097). This suggests that multiple modifiers lie within *Moe1*. 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 (Males: p > 0.713; Females: p > 0.201).

ISC x B6.Q54 Survival

We also determined the effect of ISC regions on lifespan in the F1.Q54 offspring generated by crossing ISC-A, B, C and D lines with B6.Q54. Following seizure analysis described above, F1.Q54 mice were monitored for survival to 12 weeks of age. We compared survival between F1.Q54 mice carrying B6 alleles in the ISC intervals with control heterozygous littermates using Kaplan Meier analysis. Survival to 12 weeks of age was significantly altered in females. Female mice carrying homozygous B6 alleles in the ISC-A interval had significantly reduced survival compared to heterozygous control littermates ($p < 0.004$) and ISC-B females ($p < 0.007$) (Figure 2.5, dashed). There was no significant difference in survival identified in males (Figure 2.5, solid). Within genotype groups there were sex-specific significant differences in survival. Among mice with homozygous B6 alleles in ISC-A, males lived significantly longer than females ($p < 0.009$) (Figure 2.5).

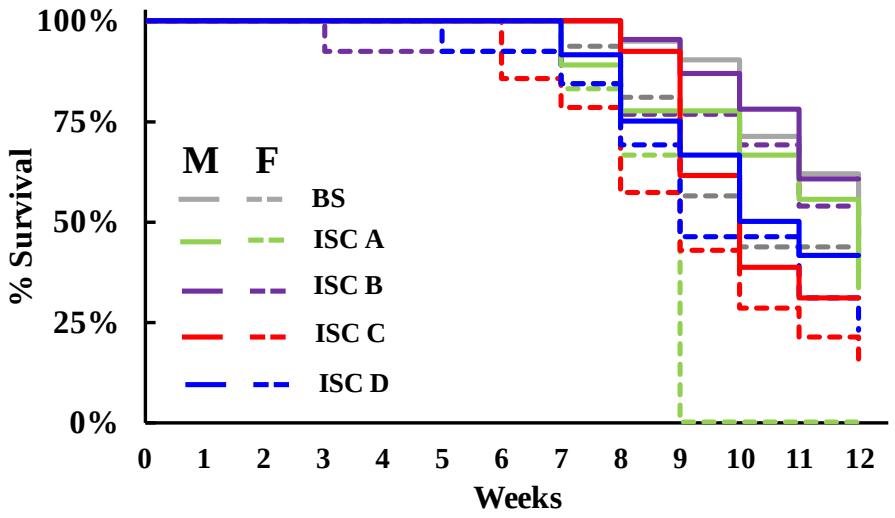

Figure 2.5 Kaplan Meier Survival Plot of ISC and Control Lines
F1.Q54 ISC-A, B, C, D and control heterozygous littermates were monitored until 12 weeks of age. ISC-A females live significantly shorter lives than control littermates ($p < 0.004$) and ISC-B females ($p < 0.007$). When comparing across sex, ISC-A males live significantly longer than ISC-A females ($p < 0.009$).

Candidate Gene Analysis by RNA-Seq

Due to the complexity of the *Moe1* interval and the high gene density of chromosome 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 (Table 2.2). 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 three genes with significant gene or transcript expression differences between B6 and SJL males. On the basis of our female ISC experiments, B6 homozygosity in the ISC-C interval (93–104 Mb) increased seizure frequency. In the ISC-C region, RNA-Seq analysis revealed 32 genes with significant gene or transcript expression differences between B6 and SJL females. A complete list of significant differences in the *Moe1* region by RNA-Seq is provided in Table 2.2.

Table 2.2 Significant Differences in the *Moe1* Interval Via RNA-Seq
Grey shading indicates a significant difference; Open boxes indicate no difference. Abbreviations are as follows: B = C57BL/6J, S = SJL/J, M = Male, F= Female.

		Gene				Transcript				TSS				Promoter				Splicing			
Interval	**Gene**	BM v BF	SM v SF	BM v SM	BF v SF	BM v BF	SM v SF	BM v SM	BF v SF	BM v BF	SM v SF	BM v SM	BF v SF	BM v BF	SM v SF	BM v SM	BF v SF	BM v BF	SM v SF	BM v SM	BF v SF
B	*Aatf*					■			■	■			■								
	Akap1						■	■	■		■	■	■		■	■	■			■	
	Cltc					■	■	■	■	■	■	■	■		■	■	■		■	■	■
	Dgke			■	■			■	■			■	■			■	■				
	Dhx40						■	■			■			■							
	Dusp14				■	■		■	■			■	■								
	Dynll2					■	■			■	■			■			■				
	Ggnbp2					■	■	■		■					■	■	■		■		
	Gm11478						■														

Table 2.2 (Cont.) Significant Differences in the *Moe1* Interval Via RNA-Seq

Grey shading indicates a significant difference; Open boxes indicate no difference. Abbreviations are as follows: B = C57BL/6J, S = SJL/J, M = Male, F= Female.

		Gene				Transcript				TSS				Promoter				Splicing			
Interval	**Gene**	BM v BF	SM v SF	BM v SM	BF v SF	BM v BF	SM v SF	BM v SM	BF v SF	BM v BF	SM v SF	BM v SM	BF v SF	BM v BF	SM v SF	BM v SM	BF v SF	BM v BF	SM v SF	BM v SM	BF v SF
B	*Gm11512*		■																		
	Gm11516	■																			
	Ints2			■		■	■	■	■												
	Med13					■	■														
	Msi2					■	■	■			■	■				■				■	■
	Pigw						■														
	Rffl			■	■	■	■	■	■	■	■	■	■	■		■	■			■	■
	Rpl13-ps1						■														
	Rps6kb1					■	■		■												
	Tada2a							■	■			■	■				■				
	Usp32					■	■		■												
	Vmp1					■	■	■	■												
	Znhit3		■			■	■				■										
D-C	*Gm11511*						■														
	Gm11512			■			■	■													
	Gm11516					■															
	Hlf					■	■	■	■	■			■				■			■	
	Stxbp4					■		■													
	Tom1l1					■	■		■						■				■		
C	*1110035M17Rik*				■		■		■												
	1700081L11Rik					■	■	■	■	■	■	■	■			■			■	■	■
	Aarsd1						■	■	■		■	■	■	■	■	■	■	■		■	
	Abi3			■		■		■	■												
	Acly					■	■	■			■							■		■	
	Atp5g1			■	■			■	■			■	■							■	
	Atxn7l3					■	■	■		■	■	■				■					
	Becn1		■			■	■	■	■	■	■	■		■	■	■		■	■	■	
	Cacna1g	■				■	■	■	■	■	■	■	■	■	■		■	■	■	■	■
	Cacnb1	■		■		■	■	■	■	■		■	■	■		■	■	■	■	■	■
	Ccdc56							■				■				■					
	Cdc27					■								■		■					
	Cdk5rap3					■	■	■	■		■	■	■	■	■	■	■	■	■	■	■
	Dcakd						■	■													
	Dhx58				■			■	■			■	■	■	■	■	■	■	■	■	■

Table 2.2 (Cont.) Significant Differences in the *Moe1* Interval Via RNA-Seq

Grey shading indicates a significant difference; Open boxes indicate no difference. Abbreviations are as follows: B = C57BL/6J, S = SJL/J, M = Male, F= Female.

		Gene				Transcript				TSS				Promoter				Splicing			
Interval	Gene	BM v BF	SM v SF	BM v SM	BF v SF	BM v BF	SM v SF	BM v SM	BF v SF	BM v BF	SM v SF	BM v SM	BF v SF	BM v BF	SM v SF	BM v SM	BF v SF	BM v BF	SM v SF	BM v SM	BF v SF
C	*Dnajc7*					■	■				■			■	■	■	■	■	■	■	
	Dusp3					■	■	■			■	■									
	Eftud2		■			■	■	■	■												
	Ezh1					■			■	■				■			■		■	■	
	Fam134c						■	■	■		■	■			■	■					■
	Fam171a2						■														
	Fbxl20					■	■	■		■						■			■	■	
	Gfap						■														
	Gm10039			■	■			■	■												
	Gm11518		■				■														
	Gm12359																		■		■
	Gosr2									■											■
	Gpatch8					■	■														
	Hap1	■				■	■	■		■	■	■				■					
	Hdac5					■	■	■	■	■	■	■	■	■	■	■	■	■		■	■
	Itga3					■	■	■	■	■		■								■	
	Jup					■		■	■	■		■	■							■	■
	Kat2a					■	■		■	■	■		■								
	Krt222						■				■				■						
	Lrrc46						■														
	Lsm12						■				■				■		■				
	Luc7l3					■	■	■		■	■	■			■						
	Med1					■	■	■	■												
	Med24					■	■	■	■												
	Mpp2					■	■	■	■		■								■	■	
	Mpp3					■	■	■		■	■	■			■						■
	Myst2				■	■	■	■	■	■	■		■	■	■	■	■	■	■	■	■
	Nfe2l1					■	■	■	■	■	■	■	■								
	Nme1					■	■	■			■	■									
	Npepps					■	■	■	■	■	■		■		■	■		■	■		■
	Nr1d1	■				■	■	■		■											
	Nsf					■	■	■		■	■	■	■	■				■	■		■
	Nt5c3l					■	■	■		■				■	■			■	■		
	Pcgf2					■		■	■	■		■	■	■			■			■	■
	Pdk2						■				■										
	Pip4k2b					■				■											
	Plcd3		■	■		■	■														
	Plekhh3	■		■		■	■	■		■	■	■		■	■	■	■	■	■	■	■

Table 2.2 (Cont.) Significant Differences in the *Moe1* Interval Via RNA-Seq
Grey shading indicates a significant difference; Open boxes indicate no difference. Abbreviations are as follows: B = C57BL/6J, S = SJL/J, M = Male, F= Female.

		Gene				Transcript				TSS				Promoter				Splicing			
Interval	**Gene**	BM v BF	SM v SF	BM v SM	BF v SF	BM v BF	SM v SF	BM v SM	BF v SF	BM v BF	SM v SF	BM v SM	BF v SF	BM v BF	SM v SF	BM v SM	BF v SF	BM v BF	SM v SF	BM v SM	BF v SF
C	*Plxdc1*			■			■	■													
	Pnpo		■	■			■	■			■	■		■	■		■	■			■
	Rab5c						■		■												■
	Slc25a39					■	■	■	■	■	■	■			■	■		■	■	■	■
	Smarce1					■	■		■	■	■			■			■				
	Snx11																■				
	Sp2						■	■	■		■	■	■	■			■				
	Srcin1						■		■		■										■
	Tbkbp1					■	■	■	■	■	■	■	■								
	Ubtf					■	■	■	■	■	■	■	■	■							
	Xylt2					■		■													
	Zfp385c						■	■			■	■		■	■	■					

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 non-synonymous (NS) SNPs in the gene *Tom1l1*. 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 Table 2.3.

Table 2.3 Non-Synonymous Coding SNPS in *Moe1*

SNPs were uploaded to Ensembl's variant call predictor to reveal functional consequences of variants. NS amino acid substitutions were uploaded to SIFT to predict the potential impact on protein function.

Interval	Gene	Location	Amino Acid Change	SNP	RS #	Sift Prediction
B	Zfp830	11:82578439	R/K	aGg/aAg		TOLERATED with a score of 0.95
	Lig3	11:82604104	R/S	agG/agT	rs28225588	TOLERATED with a score of 1
	Lig3	11:82609212	K/R	aAa/aGa	rs28225573	TOLERATED with a score of 0.26
	Lig3	11:82610931	V/D	gTt/gAt	rs28225563	TOLERATED with a score of 1
	Lig3	11:82613102	N/S	aAt/aGt	rs28225556	TOLERATED with a score of 1
	Rad51i3	11:82695305	V/A	gTc/gCc	rs28209138	AFFECT PROTEIN FUNCTION with a score of 0.02
	Nle1	11:82718877	V/I	Gtt/Att	rs13468707	TOLERATED with a score of 1
	Dusp14	11:83862583	W/R	Tgg/Cgg	rs13459132	TOLERATED with a score of 0.59
	Ints2	11:86031421	M/I	atG/atA	rs29431276	TOLERATED with a score of 0.07
	Ints2	11:86064535	R/H	cGt/cAt	rs29420094	TOLERATED with a score of 1
	Tubd1	11:86374767	T/S	Aca/Tca	rs27026986	TOLERATED with a score of 0.96
	Bzrap1	11:87575147	G/R	Ggg/Agg	rs51462366	TOLERATED with a score of 0.64
	Bzrap1	11:87589796	M/L	Atg/Ctg	rs28278310	TOLERATED with a score of 0.34
	Bzrap1	11:87591464	A/S	Gcg/Tcg	rs28278307	TOLERATED with a score of 0.75
	Lpo	11:87623710	Y/S	tAc/tCc	rs13472575	TOLERATED with a score of 0.46
	Mks1	11:87670369	K/R	aAg/aGg	rs51587946	TOLERATED with a score of 1
	Vezf1	11:87886991	S/A	Tca/Gca	rs29434284	TOLERATED with a score of 0.89
	Cuedc1	11:87998174	N/Y	Aat/Tat	rs28240982	TOLERATED with a score of 0.07
	Akap1	11:88705691	M/I	atG/atC	rs29403244	TOLERATED with a score of 0.21
	Akap1	11:88706130	T/I	aCc/aTc	rs27116277	TOLERATED with a score of 0.06
	Akap1	11:88706174	F/L	ttT/ttG	rs27116276	TOLERATED with a score of 0.75
	Akap1	11:88706910	C/S	tGt/tCt	rs27116275	TOLERATED with a score of 0.84
	Coil	11:88842523	L/S	tTg/tCg	rs29403395	TOLERATED with a score of 0.92
	Coil	11:88843369	G/D	gGc/gAc		AFFECT PROTEIN FUNCTION with a score of 0.03
D-C	Tom1l1			aCt/aTt	rs13469307	TOLERATED with a score of 0.27
	Tom1l1			gCt/gGt	rs13469308	TOLERATED with a score of 0.08
C	Samd14	11:94886027	N/H	Aac/Cac	rs27101631	TOLERATED with a score of 0.09
	Ube2z	11:95926522	S/A	Tcg/Gcg		TOLERATED with a score of 0.38
	Cdk5rap3	11:96770100	T/M	aCg/aTg		TOLERATED with a score of 0.10
	Sp2	11:96822911	T/I	aCc/aTc	rs3708840	TOLERATED with a score of 0.62
	Arhgap23	11:97309791	I/V	Atc/Gtc	rs45809946	TOLERATED with a score of 0.80
	Cwc25	11:97614056	R/W	Cgg/Tgg	rs27089733	DAMAGING with a score of 0.01
	Plxdc1	11:97802222	A/E	gCg/gAg	rs27073969	TOLERATED with a score of 0.31
	Plxdc1	11:97817828	Q/R	cAa/cGa	rs27057105	TOLERATED with a score of 0.53
	Nr1d1	11:98631530	R/Q	cGg/cAg	rs27041024	TOLERATED with a score of 0.29
	Rapgefl1	11:98708468	R/P	cGg/cCg		TOLERATED with a score of 0.15
	Top2a	11:98855618	P/L	cCt/cTt		TOLERATED with a score of 0.19
	Top2a	11:98855703	T/A	Act/Gct	rs27025992	TOLERATED with a score of 0.61
	Krt10	11:99250636	L/R	cTa/cGa		AFFECT PROTEIN FUNCTION with a score of 0.00
	Fkbp10	11:100284113	G/S	Ggc/Agc		AFFECT PROTEIN FUNCTION with a score of 0.00
	Nt5c3l	11:100291151	C/Y	tGc/tAc		TOLERATED with a score of 1
	Nt5c3l	11:100291164	Y/H	Tac/Cac	rs29442152	TOLERATED with a score of 0.55
	Nt5c3l	11:100301235	C/F	tGt/tTt		TOLERATED with a score of 0.72
	Cnp	11:100441755	D/G	gAt/gGt		AFFECT PROTEIN FUNCTION with a score of 0.03
	Zfp385c	11:100490929	A/T	Gct/Act	rs27090504	TOLERATED with a score of 0.47
	Ramp2	11:101107793	P/T	Ccg/Acg		TOLERATED with a score of 0.25
	Wnk4	11:101136570	A/S	Gct/Tct	rs52181149	TOLERATED with a score of 1
	Aarsd1	11:101278496	S/N	aGc/aAc	rs13468999	TOLERATED with a score of 0.51
	Rundc1	11:101292783	V/A	gTg/gCg	rs27057199	TOLERATED with a score of 0.59
	Rundc1	11:101294928	H/P	cAc/cCc		TOLERATED with a score of 1
	Rundc1	11:101295155	L/I	Ctt/Att	rs6402514	TOLERATED with a score of 1
	Rundc1	11:101295452	V/M	Gtg/Atg		TOLERATED with a score of 0.07
	Etv4	11:101632794	I/F	Att/Ttt	rs28234792	TOLERATED with a score of 0.71
	Tmub2	11:102149413	S/N	aGc/aAc	rs29484653	TOLERATED with a score of 0.49
	Adam11	11:102634721	Q/K	Cag/Aag		TOLERATED with a score of 0.91

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 2.4). Top candidate modifier genes were identified as *Hlf* in males and *Cacna1g* and *Cacnb1* in females.

Table 2.4 Top Candidate Modifier Genes in *Moe1*

[†]Priority rating is based on biological function and prior association with neuronal hyperexcitability phenotypes. *** highest priority; **moderate priority; *low priority.

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

In the D–C interval (89–93 Mb) that contains the 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 (35). 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 (36). A significant difference in a *Hlf* transcript was only identified between B6

and SJL males, consistent with a male-specific effect (Figure 2.6). Taken together, this suggests that *Hlf* is a strong candidate modifier gene in males (Table 2.4).

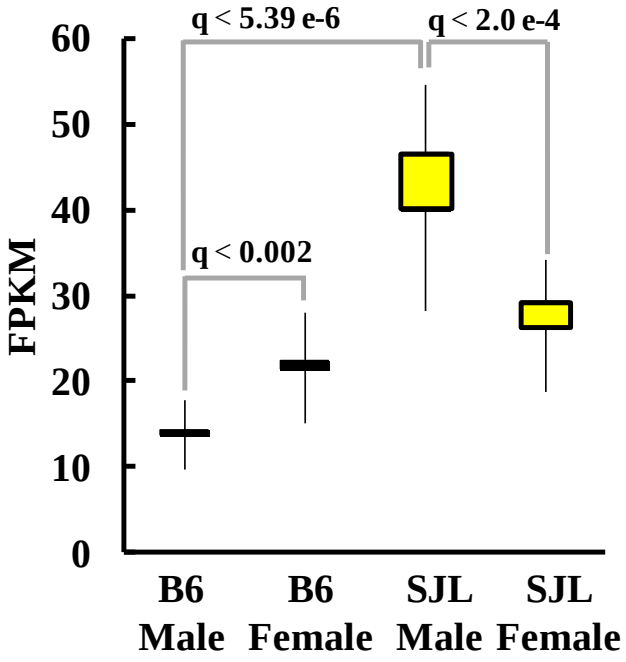

Figure 2.6 Expression of *Hlf-002* Transcript in B6 and SJL Brain
The RNA-Seq FPKM values and confidence interval for each group is shown. Boxes represent the range of values and whiskers represent the 95% confidence interval calculated by Cuffdiff. Q-values are false discovery rate-adjusted p-values (*Hlf-002*= ENSMUST00000043658).

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) (37). A family-based study of epilepsy with suggestive linkage to chromosome 17q12-24 showed differential expression of *SLC25A39* in affected family members (38). However, sequencing did not reveal a pathogenic variant in this gene. On the basis of its tenuous association with epilepsy, *Slc25a39* was retained as candidate modifier gene with lower priority (Table 2.4).

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 (39-41). Differences in five major *Cacna1g* transcripts were identified between B6 and SJL females (Figure 2.7). These transcripts, differing in splicing of exons 14, 25 and 26, have known differences in gating, kinetics and voltage dependencies (42-44). 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 (Figure 2.8). There were also several significant differences in promoter usage, TSS and splice variants between sex and strain.

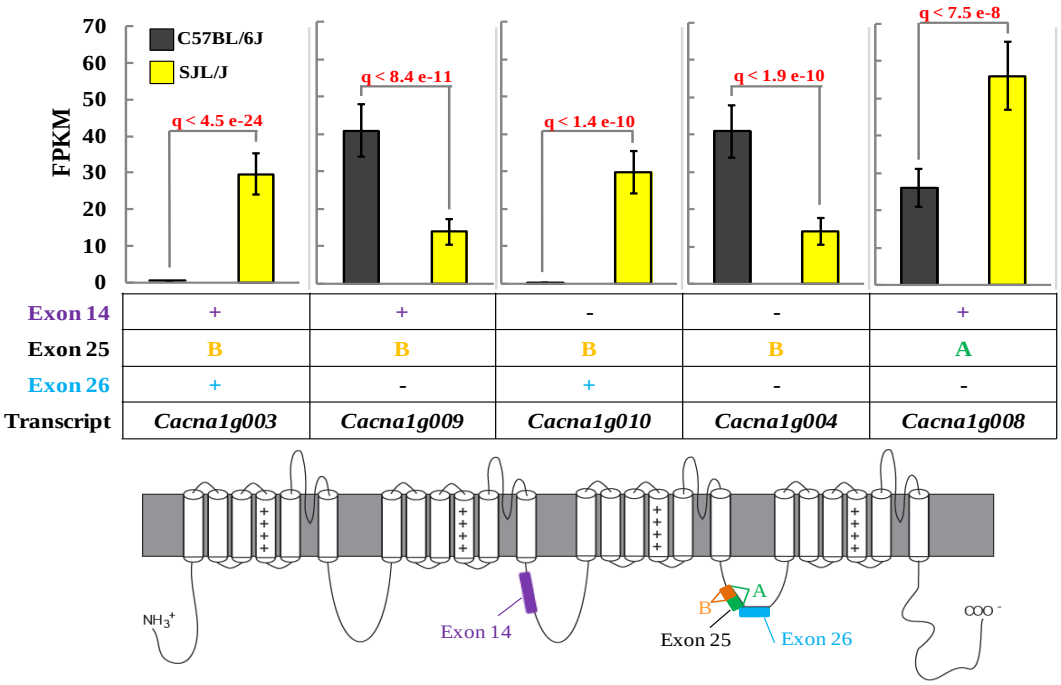

Figure 2.7 Differential Expression of *Cacna1g* Alternatively Spliced Transcripts
Inclusion/exclusion of cassette exons 14 and 26 and alternate splicing of exon 25 are shown for the five transcripts that exhibited significant differences between B6 and SJL females. The location of alternatively spliced 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.

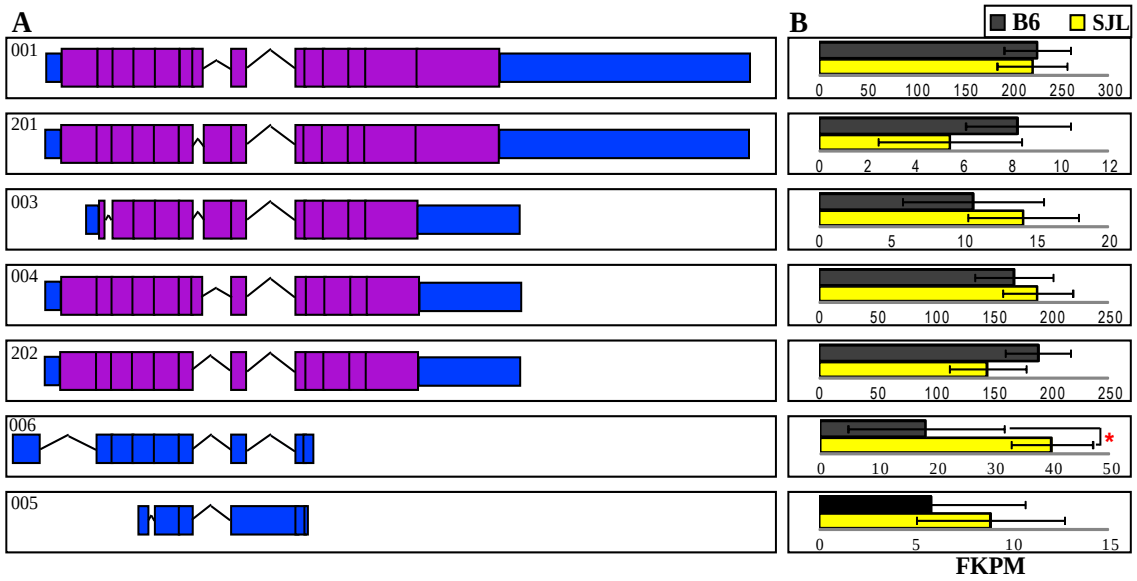

Figure 2.8 Alternative Splicing and Differential Expression of *Cacnb1*
A. *Cacnb1* alternatively spliced transcripts are shown. Purple shaded boxes represent coding regions and blue shaded boxes are non-coding regions. **B.** Respective FPKM values and confidence intervals for B6 and SJL female transcripts are shown (*q < 0.02). Q-values are false discovery rate-adjusted p-values.

Discussion

Genetic modifier loci influencing the Q54 epilepsy phenotype were initially mapped to chromosomes 11 (*Moe1*) and 19 (*Moe2*) in a backcross between B6 and SJL strains (18). We constructed ISC strains to confirm the presence of *Moe1* on chromosome 11 and refine the critical interval. Our results revealed that the interval likely contains multiple modifiers that contribute to phenotype severity of Q54 mice. Sex specific modifiers were also identified within the interval. Similar to our results, previous studies of epilepsy quantitative trait loci (QTL) in polygenic mouse models have shown that the substructure of QTL can be complex with multiple genes contributing to the low-resolution mapping peak (14, 45).

In females, B6 homozygosity in the ISC-C interval conferred increased seizure frequency, suggesting the presence of a permissive modifier gene in this interval. The large ISC-A region that spans the entire *Moe1* 1 LOD support interval resulted in females with near-significant differences in seizure frequency ($p > 0.097$) and reduced survival ($p < 0.004$) compared to heterozygous controls. This suggests that significant differences in seizure frequency in ISC-A would likely result if more females were added to the study. Significant seizure frequency differences in ISC-A further supports *Moe1* as a modifier locus of the Q54 phenotype.

In males, increased seizure frequency was associated with B6 homozygosity in the ISC-D interval but not in the ISC-C interval. 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). Interestingly, ISC-A males did not experience significant differences in seizure frequency compared to heterozygous controls ($p > 0.312$). This

suggests that multiple modifiers lie within *Moe1* which affect the male Q54 phenotype. B6 homozygosity in the proximal region of the *Moe1* interval may mask the effect of more distal permissive modifiers in males. However, B6 homozygosity in the small ISC-B proximal interval (81–93 Mb) alone does not significantly reduce male seizure frequency compared to heterozygous controls ($p > 0.713$).

Utilizing RNA-Seq, we were able to identify a significant number of transcripts that exhibited within-strain sex differences throughout the *Moe1* interval. These differences likely result from multiple different causes, which may include brain sexual dimorphism, epigenetics and endocrine influences.

Combined ISC line seizure data and RNA-Seq analysis identified several strong candidate modifiers that may contribute to severity of the Q54 seizure phenotype. Top candidate modifier genes include *Cacna1g* and *Cacnb1* in females and *Hlf* in males. Although we identified significant transcript differences in *Cacna1g, Cacnb1* and *Hlf*, 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 calcium (Ca^{2+}) channel α_1 subunit which propagates low-voltage activated (LVA) Ca^{2+} currents (46). T-type channels regulate neuronal firing by conducting Ca^{2+} during action potentials and can switch neurons between firing modes (47). 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 (48, 49). 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 (50-52). 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 (53). Deletion of *Cacna1g* in mice suppressed $GABA_B$ receptor agonist-induced SWDs, indicating an essential role for this gene in seizure expression (52, 54). In humans, SNPs in *CACNA1G* have been associated with idiopathic generalized epilepsy (41).

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 (42, 43). Inclusion of cassette exon 26 contributes to more hyperpolarized steady-state inactivation and slower recovery from inactivation (42). Exon 25 has an internal donor site, resulting in a short exon 25 (B) or full length (A) (Figure 2.7) (42-44, 55). Transcripts with the 25A exon activate and inactivate at more hyperpolarized potentials, while 25B exon transcripts have slower activation and inactivation kinetics (42). On the basis of 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 (Figure 2.7). 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 (50-52). 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 (Figure 2.7) (42). 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 (56).

Cacnb1 encodes one of four homologous Ca^{2+} channel β subunits. β subunits modulate α_1 channel trafficking, gating and kinetics (57-59). *Cacnb1* has four splice variants, which are highly expressed in the hippocampus and dentate gyrus (57, 58). 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 (39, 60). In humans, surgical resection samples from temporal lobe epilepsy patients exhibited an upregulation of *CACNB1* and *CACNB2* (59). 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 (59, 61-63). *CACNB1* mutations have been identified in patients with a history of febrile seizures, juvenile myoclonic epilepsy, episodic ataxia, generalized epilepsy and praxis-induced seizures (39, 40).

RNA-Seq analysis determined that SJL females express significantly higher levels of transcript *Cacnb1*-006 than B6 females (Figure 2.8). 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 shows the importance of non-coding RNAs (ncRNA) in the brain (64-68). ncRNAs have been shown to contribute to neural differentiation and cell identity, hippocampal development, oligodendrocyte myelination and synaptic plasticity (69). Additionally, lncRNAs have been implicated in neurodevelopmental, neurodegenerative, neuroimmunological and psychiatric disorders and, relevant to our studies, epilepsy (69). Mice deficient in the lncRNA *Evf2* had reduced numbers of GABAergic interneurons in the hippocampal formation and displayed reduced synaptic inhibition (70). 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*$^{KO/KO}$ homozygous knockout mice exhibited audiogenic seizures and it is hypothesized that *BC1* lncRNA modulates hyperexcitability via the metabotropic glutamate receptor pathway (71). It is possible that the *Cacnb1*-006 transcript may act as an 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* and *Tef*) results in mice with spontaneous, generalized tonic-clonic and absence seizures (36). Microarray expression analysis performed to investigate contributors to the PAR bZIP knockout epilepsy phenotype, revealed a twofold reduction in pyridoxal kinase (PDXK). PDXK encodes a coenzyme involved in the conversion of pyridoxine to pyridoxal 5′-phosphate (PLP), a key coenzyme involved in amino acid and neurotransmitter metabolism (36, 72). Mice defective in PLP metabolism experience fatal seizures, found to be a consequence of significantly reduced GABA levels (73). Mice maintained on a pyridoxine deficient diet, resulting in reduced PLP levels, experienced increased audiogenic seizures (74-76).

Human pyridoxine-dependent epilepsies can be successfully treated with PLP (77). Our RNA-Seq analysis showed that B6 males have a significant reduction in *Hlf* expression compared to SJL males (Figure 2.6). 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 pyridoxine deficiency on B6.Q54 mice may help determine the contribution of this pathway to Q54 epilepsy severity.

On the basis of 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 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 patients.

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CHAPTER III

VALIDATION OF *CACNA1G* AND *HLF* AS GENETIC MODIFIERS OF THE *SCN2A*^{Q54} EPILEPSY PHENOTYPE

Introduction

Epilepsy is a common neurological disorder affecting approximately 3 million people in the United States (1). Nearly 900 mutations identified in voltage-gated sodium channel genes result in several types of human epilepsies, including Genetic Epilepsy with Febrile Seizures Plus (GEFS+), Benign Familial Neonatal-Infantile Seizures (BFNIS), Dravet and Ohtahara syndromes (2-4). Among affected family members who possess the same sodium channel mutation, the clinical severity of their disease can be strikingly different. This suggests that other factors, possibly genetic, must modify the primary mutation, resulting in a more or less severe phenotype.

Several mouse models have been generated in order to study the genetic basis of epilepsy. A common feature found in these models is that the genetic strain background alters the disease phenotype, suggestive of genetic modifiers in epilepsy. The *Scn2a*^{Q54} (Q54) transgenic mouse model has a gain-of-function mutation that results in persistent sodium current and epilepsy (5). On the C57BL/6J (B6) strain background, B6.Q54 mice have infrequent, adult-onset focal motor seizures and a 75% survival rate beyond six months of age (6). When crossed with the SJL/J (SJL) strain, the resulting F1.Q54 generation experience high seizure frequency with juvenile onset and less than 25% survival until six months of age (6). We mapped two dominant loci that modify seizure susceptibility in Q54, designated *Moe1* (Modifier of Epilepsy) on chromosome 11 and

Moe2, on chromosome 19 (6). *Kcnv2* was identified as the modifier gene at *Moe2* (7). Recent fine-mapping and RNA-Seq data suggested two sex-specific candidate modifier genes at the *Moe1* locus, *Cacna1g* and *Hlf* in females and males, respectively (8).

Cacna1g encodes the T-type calcium channel α_1 subunit that propagates low-voltage activated (LVA) calcium currents (9). Several mouse models that exhibit abnormalities in LVA calcium currents exhibit absence seizures and abnormal thalamocortical synchronization (10). Furthermore, single-nucleotide polymorphisms in human *CACNA1G* have been identified in patients with generalized epilepsy (11). *Cacna1g* has twelve identified alternative splice sites, resulting in numerous transcripts with distinct current properties (9, 12-15). *Cacna1g* is hypothesized to modify the Q54 seizure phenotype through significant strain-specific differences in expression profiles of transcripts that have functional kinetic diversity in their LVA currents (8). SJL female mice preferentially express *Cacna1g* channels that reduce the likelihood of synchronous thalamocortical oscillations, a common feature of epilepsy (8, 16-18). In contrast, B6 female mice express *Cacna1g* channels that are prone to hyperexcitability (8).

Hlf encodes hepatic leukemia factor, a member of the PAR bZIP transcription factor family. Deletion of the PAR bZIP transcription factor family (*Hlf*, *Dbp* and *Tef*) in a knock-out mouse model resulted in mice with spontaneous, generalized tonic-clonic and absence seizures (19). Microarray expression analysis was utilized to determine contributors to the epilepsy phenotype (19). A two-fold reduction in pyridoxal kinase (PDXK), a key enzyme involved in the conversion of pyridoxine to pyridoxal 5' phosphate (PLP) was discovered (19). PLP is a key coenzyme involved in amino acid and neurotransmitter metabolism, including GABA and glutamate (Figure 3.1) (20).

Interestingly, PLP has a substantial connection to epilepsy. Pyridoxine-dependent epilepsies are successfully treated with PLP (21). Mice maintained on a pyridoxine deficient diet, consequently reducing PLP levels, experienced increased seizure frequency (22-24). Furthermore, mice defective in PLP metabolism experienced fatal seizures (25). *Hlf* is hypothesized to modify the Q54 seizure phenotype possibly by altering PDXK and PLP levels in a strain-dependent manner (Figure 3.1) (8). In males, B6 express *Hlf* at a significantly reduced level compared to SJL (8). A reduction in *Hlf*, with consequent reductions in PDXK and PLP levels, may be responsible for the increased seizure susceptibility seen in B6 males (8).

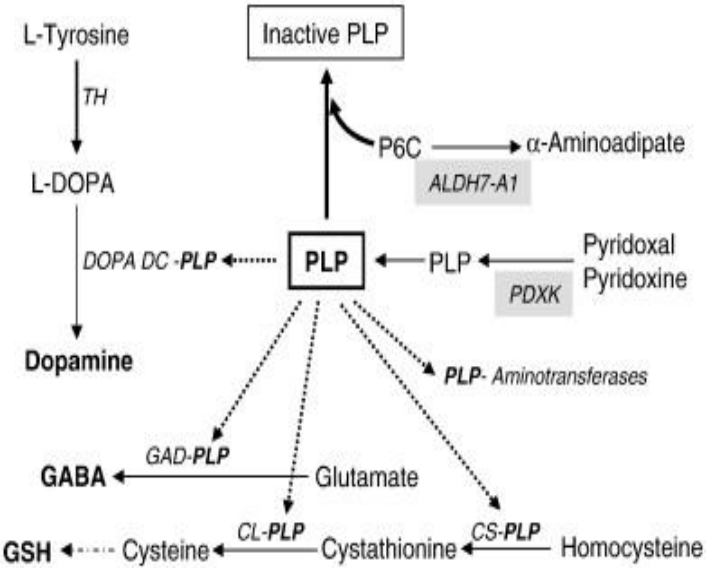

Figure 3.1 The Pyridoxine Pathway
Diagram illustrates the involvement of PDXK, PLP and pyridoxine in neurotransmitter metabolism (26). When the PAR bZIP family is knocked out, including *Hlf*, PDXK levels are reduced two-fold (19). Reprinted with permission (26).

To determine the contribution of *Cacna1g* and *Hlf* to the seizure phenotype of Q54 mice, we utilized *Cacna1g* BAC transgenic and *Hlf* targeted knockout mouse models. We systematically evaluated the candidate modifier genes by testing their ability

to enhance or reduce severity of the Q54 seizure phenotype in double mutant mice. In addition to seizure analysis, we also evaluated our *Hlf* hypothesis by testing the effects of pyridoxine deficient and enriched diets on the Q54 phenotype. Furthermore, to determine whether *Hlf* would modify other types of epilepsy, we assessed the ability of *Hlf* to modify the Dravet phenotype of *Scn1a*$^{KO/+}$ heterozygous knockout mice.

Materials and Methods

Mice

A BAC clone containing *Cacna1g* (RP23-65I14) was obtained from BACPAC Resources (http://bacpac.chori.org/home.htm). BAC DNA was prepared with the NucleoBond BAC 100 kit, according to manufacturer's instructions (Macherey-Nagel). DNA was reconstituted in polyamine injection buffer (27). Pronuclear injections into SJL fertilized oocytes were performed by the Vanderbilt University Transgenic Mouse/ESC Shared Resource. Injections resulted in a single *Cacna1g* BAC transgenic founder. The resulting strain, 1G5, is maintained on the SJL strain by continued backcrossing of hemizygous males to SJL females (Jackson Labs stock #000686).

Hlf ^{tm1Schb} *(Hlf* $^{KO/+}$*)* targeted knockout embryos congenic on B6 were obtained from the European Mouse Mutant Archive *(*www.emmanet.org) (19). Fifty *Hlf* $^{KO/+}$ heterozygous knockout embryos were implanted into pseudopregnant females at the Vanderbilt Transgenic Mouse/ESC Shared Resource. The *Hlf* $^{KO/+}$ heterozygous knockout line is maintained on the B6 strain by continued backcrossing of B6.*Hlf* $^{KO/+}$ males to B6 females (Jackson Labs stock #000664).

Q54 transgenic mice [Tg(Eno2-Scn2a1*)Q54Mm] were generated as previously described (5). The B6.Q54 line is maintained on the B6 strain by continued backcrossing of hemizygous B6.Q54 males to B6 females (Jackson Labs stock #000664). *Scn1a*^{tm1Kea} (*Scn1a*$^{KO/+}$) heterozygous knockout mice were generated as previously described (28). The *Scn1a*$^{KO/+}$ line is maintained on the 129S6/SvEvTac (129) strain by continued backcrossing of 129.*Scn1a*$^{KO/+}$ males to 129 females (Taconic model #129SVE).

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.

Genotyping

Mice were tail biopsied on postnatal day 14 (P14) and genotyped. DNA was prepared from tail biopsies using the Gentra Puregene Mouse Tail Kit according to the manufacturer's instructions (Qiagen). 1G5 transgenics were identified by using the 1G SP6 and 1G T7 primers indicated (Table 3.1). These primers amplify a 200 bp and 220 bp product, respectively. *Hlf* knockout mice were genotyped using primers designed to target the LacZ cassette. Primers *LacZ R*: 5'ATGTGAGCGAGTAACAACCCGTCGGA TCT, *Hlf_LF*: 5'CCCTTTTTCTTCTTCAGCATTTAG and *Hlf_R*: 5'GGATGCCATTC TCTGACAGG amplify a 200 bp wildtype product and a 400 bp knockout product. The Q54 transgene was genotyped as previously described (5). *Scn1a* knockouts were genotyped as previously described (28). All genotyping was performed by PCR and analyzed on 2% agarose gels.

BAC Integrity, Copy Number and Expression

BAC integrity was evaluated in genomic DNA by PCR amplification of microsatellite and vector markers spanning the BAC (Table 3.1). SP6 and T7 vector markers flanked each end of the BAC construct, while D11VU microsatellite markers were located approximately every 32 Kb, including two within *Cacna1g* (Figure 3.2).

To assess copy number, we used a Taqman assay as described previously by Chandler, *et al* (29). Quantitative PCR (qPCR) was performed using custom-designed *Cacna1g* and reference *Scn5a* Taqman assays described previously by Ernst, *et al* (Table 3.1) (18). Genomic DNA from transgenic (n=17), homozygous transgenic (n=6) and control (n=8) littermates was used as template and a standard curve was applied to estimate copy number. Copy number standards were prepared by spiking SJL genomic DNA with BAC plasmid DNA equivalent to 0, 5, 10 and 20 copies per diploid genome.

Transcript expression analysis was performed using Taqman gene expression assays (Applied Biosystems) (Table 3.1). RNA was extracted from whole brains of six week old transgenic (n=15), homozygous transgenic (n=7) and control (n=10) littermates using the RNeasy kit (Qiagen). Total RNA was then reverse transcribed using the SuperScript III First-Strand Synthesis System according to the manufacturer's instructions (Invitrogen). Samples were run in triplicate on an ABI 7900 HT and replicated over four plates. Relative transcript levels were assessed using the ΔΔCT method (30). Expression differences were compared between groups by ANOVA with Fisher's PLSD *post hoc* tests.

Table 3.1 Primer Sequences for *Cacna1g* BAC Evaluation

Evaluation of the BAC construct was completed through BAC integrity, copy number and expression analyses. BAC integrity was determined using the microsatellite markers indicated which spanned the entire BAC construct. Copy number was assessed by Taqman assay on genomic DNA. Expression analysis was performed on whole brain RNA using Taqman gene expression assays.

BAC Integrity		
Primer Name	**Forward Sequence**	**Reverse Sequence**
D11VU2	TCCCATGGAGCATTGAAATC	AGAGGAAGCTAGCCCCAGAG
D11VU3	TTTCCCGATCACCAAGAGAC	GACCCTCAGCTAGGCAGTTG
D11VU5	CTGGATATTTCCCCCTGGAG	CGGTTCTTCCAGGACAGGTA
D11VU8	TTCTGCCCTCCCAGAGATAA	TCCTCAGGAGGGGATATCGT
D11VU11	TGATCCCCCTGCATAACACT	TGGTGAGCCTCTGAAGGTTT
D11VU14	GAAGCAGCCAGGTTTCTCAC	TTCCAGTGGCAAACACCATA
D11VU15	TTTACATTTTGACCAGCAAATAGC	TCAGCCAGCCTAGTTGAACA
D11VU17	TACCCTTTGCCACGATAAGG	GAGGCACTTCCAACCTTTCA
1G T7	TAATACGACTCACTATAGGG	ATGGAGTGGACACATTGCAG
1G SP6	GATTTAGGTGACACTATAG	TTCTAGGGACGTCAGGAAGC
Copy Number		
Assay Name	**Forward / Taqman Assay Sequence**	**Reverse Taqman Assay Sequence**
Cacna1g	TGGAACTGCCCATCATGAGAT	CCTCATTCTGCTGTCCTGCTAAT
Scn5a	AGGGTTTCTCTGTGACCCAAAC	AGAGCTACGGGACACAGTATCCA
TaqM_*Cacna1g*	5'-6FAM-ATCACCGGTAAGGGAATGCATGCCAC-TAMRA-3'	
TaqM_*Scn5a*	5'-6FAM-CTTCCCCTACCCTTTTCCAGGCTCTCA-TAMRA-3	
Expression		
Assay Name	**Taqman Assay**	
Cacna1g	Mm00486571_m1	
Tbp	Mm00446971_m1	

Generation of Double Mutant Mice

Cacna1g double mutant mice were generated by crossing SJL.1G5 females with B6.Q54 males. The resulting F1 generation had F1.1G5;Q54 mice and single mutant littermate controls at the expected Mendelian ratios.

Hlf double mutant mice were generated on two background strains. First, B6.*Hlf* $^{KO/+}$ heterozygous females were crossed with B6.Q54 males to generate heterozygous B6.*Hlf* $^{KO/+}$;Q54 offspring. To produce animals for phenotyping experiments, B6.*Hlf* $^{KO/+}$;Q54 males were crossed with either B6.*Hlf* $^{KO/+}$ or SJL females. Crossing B6.*Hlf* $^{KO/+}$;Q54 with B6.*Hlf* $^{KO/+}$ resulted in homozygous knockout, *Hlf* $^{KO/KO}$;Q54 and heterozygous knockout B6.*Hlf* $^{KO/+}$;Q54 test mice and control

littermates. Offspring from this cross were not obtained at the expected Mendelian ratios (See Results section). Crossing B6.*Hlf* $^{KO/+}$;Q54 with SJL resulted in F1 double mutant F1.*Hlf* $^{KO/+}$;Q54 offspring and control F1.Q54 and F1.*Hlf* $^{KO/+}$ littermates that were obtained at the expected Mendelian ratios.

To assess if *Hlf* was able to modify the Dravet phenotype of *Scn1a*$^{KO/+}$ mice, B6.*Hlf* $^{KO/+}$ females were crossed with 129.*Scn1a*$^{KO/+}$ males. The resulting F1 generation produced double mutant F1.*Hlf* $^{KO/+}$;*Scn1a*$^{KO/+}$ and single mutant littermate controls at the expected Mendelian ratios.

Phenotyping

Experimental mice were phenotyped as previously described (8). Briefly, littermates underwent 30 minute video-taped observations at three and six weeks of age. All observation sessions occurred between 1:00 PM and 4:00 PM. On the basis of prior extensive video-electroencephalogram monitoring, spontaneous behavioral seizures were assessed offline by a blinded observer using Observer XT software (Noldus) (5). We counted the number of visible focal motor seizures with forelimb clonus and repetitive movements lasting one to five seconds (Racine seizure scale 3) (31). Seizure counts from the three and six week observations were combined to obtain a frequency of seizures in 60 minutes for each animal. Other seizure types, including generalized tonic-clonic seizures (GTCS), were also counted if observed.

Pyridoxine Deficient and Enriched Diets

B6.Q54, F1.Q54, B6.*Hlf* $^{KO/+}$, B6.*Hlf* $^{KO/KO}$, B6.*Hlf* $^{KO/KO}$;Q54 and wildtype littermates were maintained on pyridoxine deficient (AIN-93G/No added pyridoxine), pyridoxine enriched (AIN-93G/8250 ppm added pyridoxine) or control (AIN-93G) diets (Purina Test Diet). At three weeks of age, mice were placed on the modified or control diet for six weeks. Animals were weighed and evaluated for general health once per week. At nine weeks of age, all mice underwent 30 minute video-taped observation to determine seizure frequency, as described above.

Neurochemistry

A subset of mice maintained on the deficient or control diet were used for neurochemistry studies performed at nine weeks of age (n=4 for each genotype, sex and diet). Mice were deeply anesthetized with isoflurane, decapitated and forebrain was rapidly dissected and frozen in liquid nitrogen. Neurochemistry was performed at the Vanderbilt Neurochemistry Core Laboratory to assay for biogenic amine neurotransmitters, biogenic amine metabolites, amino acids and amino acid neurotransmitters. Detection was achieved using Waters high performance liquid chromatography (HPLC) systems equipped with autosamplers and either a Decade II electrochemical (monoamines) or 474 scanning fluorescence (amino acids) detector.

Statistical Analysis

Seizure frequencies for phenotyping studies were compared between double mutant and single mutant control littermates using either analysis of variance (ANOVA)

with Fisher's protected least significant difference (PLSD) *post hoc* tests or unpaired two-tailed Student's t-test. Outlier analysis was performed and subjects with seizure frequencies two standard deviations above and below the mean at three or six weeks of age were removed. Mice with missing three or six week data due to outlier frequencies or death were not included in the study. Seizure frequencies for the pyridoxine diet studies were compared between modified and control diet mice by unpaired two-tailed Student's t-test. Survival was assessed using Kaplan-Meier analysis with Logrank Mantel-Cox p-values. Male and female data were analyzed separately, unless otherwise indicated.

Results

Cacna1g

Generation and Evaluation of Cacna1g BAC Transgenic Mice

To evaluate the contribution of *Cacna1g* to the seizure phenotype of F1.Q54 mice, we generated a *Cacna1g* BAC transgenic mouse model. Pronuclear injections of the BAC clone RP23-65I14, which contained *Cacna1g*, into SJL fertilized oocytes resulted in a single female *Cacna1g* BAC transgenic founder, designated 1G5.

Integrity of the 1G5 BAC construct was initially determined using primers designed for the SP6 and T7 ends flanking the BAC insert (Table 3.1). The 1G5 founder genotyped positive for both end markers. Since the BAC construct was derived from B6 and injected into SJL, additional genotyping was performed using microsatellite markers spanning the BAC to ensure a whole construct was present. The microsatellite markers

were located approximately every 32 Kb, including two within *Cacna1g* (Figure 3.2). All markers were found to be heterozygous, suggesting integration of an intact construct.

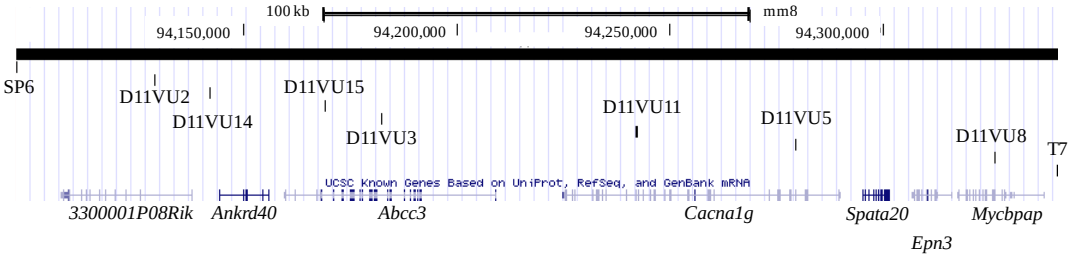

Figure 3.2 Verification of BAC Integrity
Genotyping was performed to confirm complete integration of the RP23-65I14 BAC into the SJL genome. Location of the flanking SP6 and T7 markers and the internal microsatellite markers are indicated.

After confirming the incorporation of an intact BAC construct in the 1G5 line, we assessed copy number by qPCR (29). Custom-designed *Cacna1g* and reference *Scn5a* Taqman assays were previously developed to detect the RP23-65I14 construct (18). We plotted ΔCt values from the copy number standards on the Y-axis with the known copy number plotted on the X-axis, using a linear scale. From this, we generated a linear trendline with an accompanying $y=mx+b$ slope equation (Figure 3.3). The resulting equation was used to estimate copy number of 1G5 hemizygous (n=17), homozygous (n=6) and control (n=8) mice. By fitting ΔCt values (y) from 1G5 offspring DNA, we estimated the BAC copy number (x) to be between 5-8 transgene copy integrations.

During breeding of the 1G5 N2 generation (1G5 Tg N1 male by SJL female), it became apparent that the construct integrated onto the X chromosome due to the X-linked inheritance pattern. Therefore, we intercrossed 1G5 females (N2) and 1G5 males (N1) to generate transgene positive males. Offspring from this intercross were obtained in the expected Mendelian ratios for an X-linked trait. Females had a 1:1 homozygous

(n=19) to hemizygous (n=22) ratio and males had a 1:1 hemizygous (n=22) to wildtype (n=22) ratio.

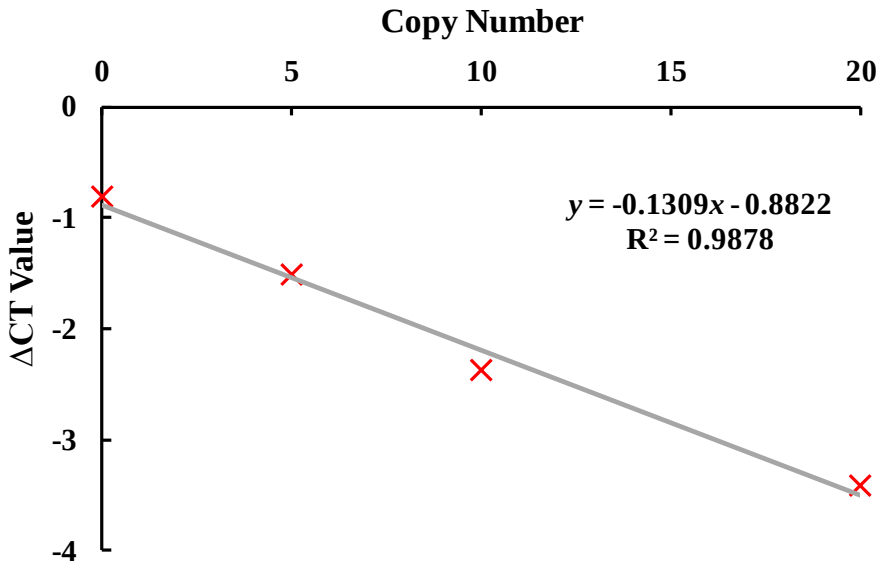

Figure 3.3 Evaluation of 1G5 Copy Number
To assess copy number, a Taqman assay was performed on genomic DNA from 1G5 offspring and controls. A standard curve was generated with 0, 5, 10 and 20 copies which revealed transgenic mice have an estimated 5-8 transgene copy integrations.

As a final evaluation of the 1G5 line, we performed transcript analysis to detect if *Cacna1g* was expressed at an increased level compared to wildtype SJL. RNA was extracted from whole brains of six week old hemizygous (Female n=8; Male n=7), homozygous (Female n=7) and control (Female n=8; Male n=2) littermates and then reverse transcribed. Expression was determined using Taqman gene expression assays for *Cacna1g* (Mm 00486571_m1) and the control *Tbp* (Mm 00446971_m1). This qPCR assay revealed only hemizygous 1G5 males had a significant increase in expression compared to wildtype ($p < 0.007$) (Figure 3.4). Hemizygous 1G5 females were not significantly different from wildtype levels. There was a significant two-fold increase in expression between homozygous 1G5 females and hemizygous 1G5 females and males

(Figure 3.4). These results suggest the 1G5 transgene may be subject to partial X-inactivation. Complete inactivation of the 1G5 transgene would result in equivalent *Cacna1g* expression levels in homozygous 1G5 females and hemizygous 1G5 males.

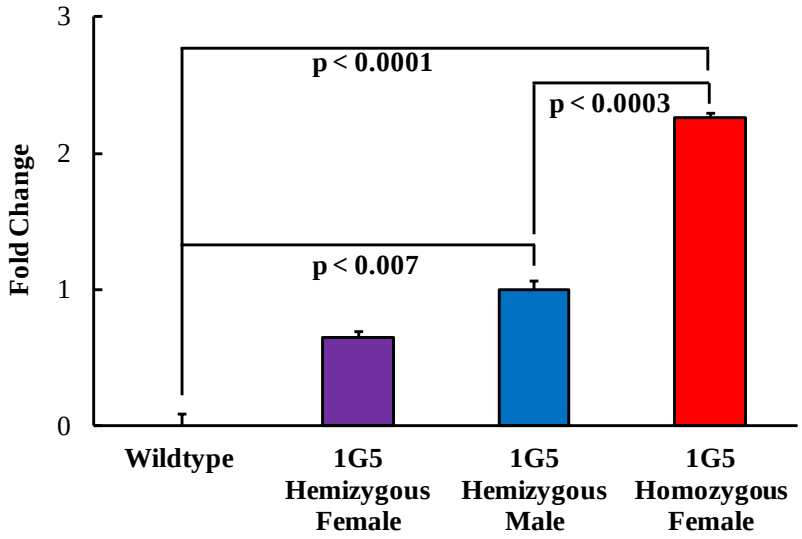

Figure 3.4 1G5 Expression Using a Taqman qPCR Assay
To assess expression, a Taqman assay was performed on 1G5 offspring and controls. Fold difference in six week old whole-brain expression of *Cacna1g* are as follows: wildtype controls= 0 ± 0.079, 1G5 hemizygous females= 0.649 ± 0.037, 1G5 hemizygous males= 1 ± 0.065 and 1G5 homozygous females= 2.26± 0.032. ANOVA $F_{(3,49)}$= 15.697, $p < 0.0001$. P-values were determined by Fishers PLSD *post hoc* test. Error bars represent SEM.

Effect of Cacna1g on F1.Q54 Phenotype

We systematically evaluated *Cacna1g* by testing its ability to enhance or reduce severity of the F1.Q54 seizure phenotype. To do this, we generated double mutant mice by crossing female SJL.1G5 with B6.Q54 males to generate F1 offspring carrying both transgenes (F1.1G5;Q54) and controls carrying either F1.Q54 or F1.1G5. Experimental mice underwent 30 minute video-taped observations for visible, spontaneous seizures at three and six weeks of age. The two time points were then combined to determine the seizure frequency in 60 minutes. Seizure analysis of F1.1G5 offspring (Male n=17;

Female n=18) revealed no apparent motor seizures. This was consistent with previous work on transgenic mice generated with the same BAC construct that exhibited frequent behavioral arrest but lacked evident motor seizures or other obvious neurological abnormalities (18). Therefore, the average seizure frequency was compared between double mutant F1.1G5;Q54 mice and single F1.Q54 controls.

Double transgenic F1.1G5;Q54 males had significantly more seizures than F1.Q54 controls ($p < 0.004$) (Figure 3.5). No difference in seizure frequency was identified between female F1.1G5;Q54 and F1.Q54 controls ($p > 0.858$) (Figure 3.6).

Following seizure phenotyping, mice from the SJL.1G5 by B6.Q54 cross were retained until 12 weeks of age to examine the effect of *Cacna1g* overexpression on the lifespan of F1.Q54 mice. The transgene had no effect on survival between F1.1G5;Q54 mice compared to F1.Q54 controls in males or females (Data not shown).

To properly evaluate the contribution of *Cacna1g* on the seizure phenotype of F1.Q54 mice, at least two separate transgenic lines are required to confirm the phenotype and avoid misinterpretation of results due to BAC insertion site effects. At this time, we were only able to generate and evaluate one *Cacna1g* BAC transgenic mouse model, designated 1G5. Results from the SJL.1G5 by B6.Q54 cross suggest *Cacna1g* is a genetic modifier of the F1.Q54 phenotype in males. We were unable to reliably determine if *Cacna1g* was a genetic modifier of the F1.Q54 phenotype in females due to the possibility that the transgene is subject to X-inactivation.

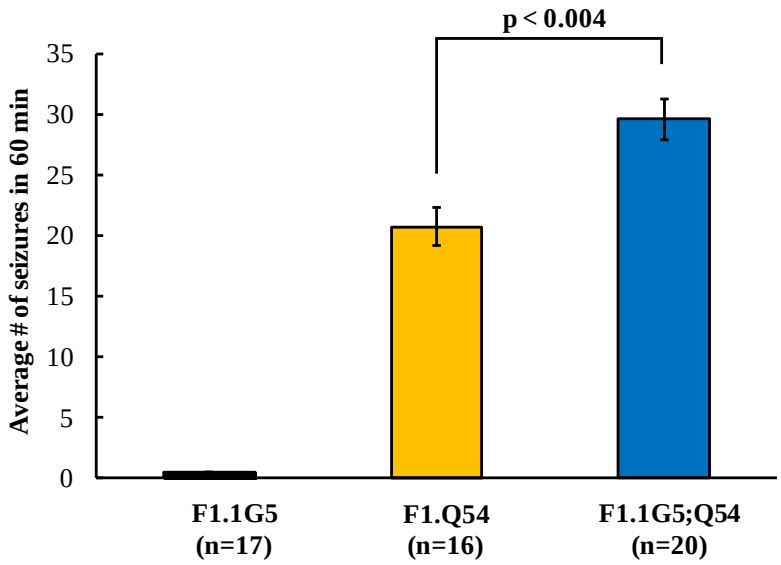

Figure 3.5 Average Seizure Frequency of SJL.1G5 X B6.Q54 Males
Average number of seizures in 60 minutes for each genotype is shown. Average seizure frequencies for males are as follows: F1.1G5= 0, F1.Q54= 22.3 ± 1.76 and F1.1G5;Q54= 29.6 ± 1.68. P-value was determined by Student's t-test. Error bars represent SEM.

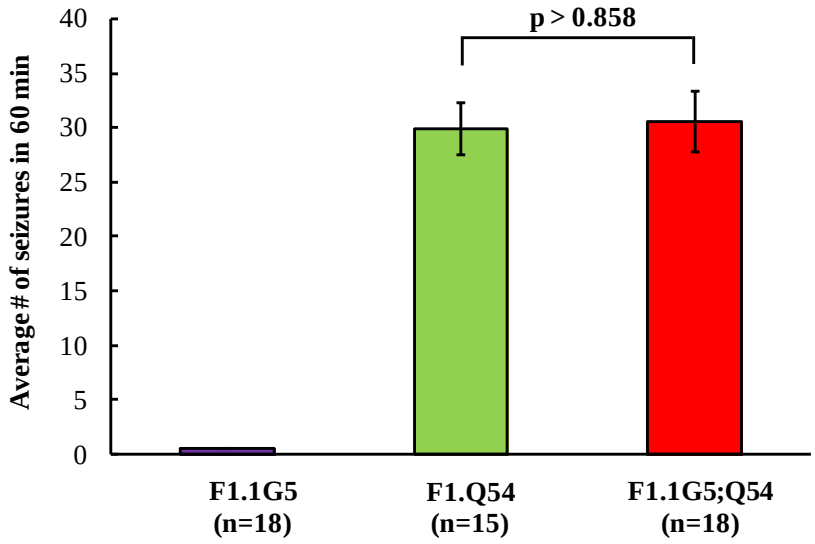

Figure 3.6 Average Seizure Frequency of SJL.1G5 X B6.Q54 Females
Average number of seizures in 60 minutes for each genotype is shown. Average seizure frequencies for females are as follows: F1.1G5= 0, F1.Q54= 30.3 ± 2.31 and F1.1G5;Q54= 32.3 ± 2.84. P-value was determined by Student's t-test. Error bars represent SEM.

Hlf

Generation of Hlf Double Mutant Mice

In order to systematically evaluate *Hlf*, we tested its ability to alter the seizure phenotype of F1.Q54 and B6.Q54 mice. To do this, we generated double mutant mice on two strain backgrounds. B6.*Hlf* $^{KO/+}$ females were first crossed with B6.Q54 males to generate B6.*Hlf* $^{KO/+}$;Q54 offspring. To generate double mutant animals on the F1 strain background, B6.*Hlf* $^{KO/+}$;Q54 males were crossed with SJL females. This cross resulted in F1.*Hlf* $^{KO/+}$;Q54 test mice and controls carrying either F1.Q54 or F1.*Hlf* $^{KO/+}$ (Figure 3.7). The resulting offspring were obtained at the expected Mendelian ratios. To generate double mutant mice on the B6 strain, we crossed B6.*Hlf* $^{KO/+}$;Q54 males with B6.*Hlf* $^{KO/+}$ females which resulted in homozygous knockout B6.*Hlf* $^{KO/KO}$;Q54 and heterozygous knockout B6.*Hlf* $^{KO/+}$;Q54 test mice and control littermates carrying B6.*Hlf* $^{KO/+}$, B6.*Hlf* $^{KO/KO}$, or B6.Q54 (Figure 3.7). Offspring from this cross were not obtained at the expected Mendelian ratios. While no difference was identified in males, female genotype ratios were significantly different (χ^2= 17.836, $p < 0.003$) (Table 3.2). More specifically, there was a reduction in female B6.*Hlf* $^{KO/KO}$;Q54 and B6.*Hlf* $^{KO/+}$ births and an increase in B6.*Hlf* $^{KO/+}$;Q54 births.

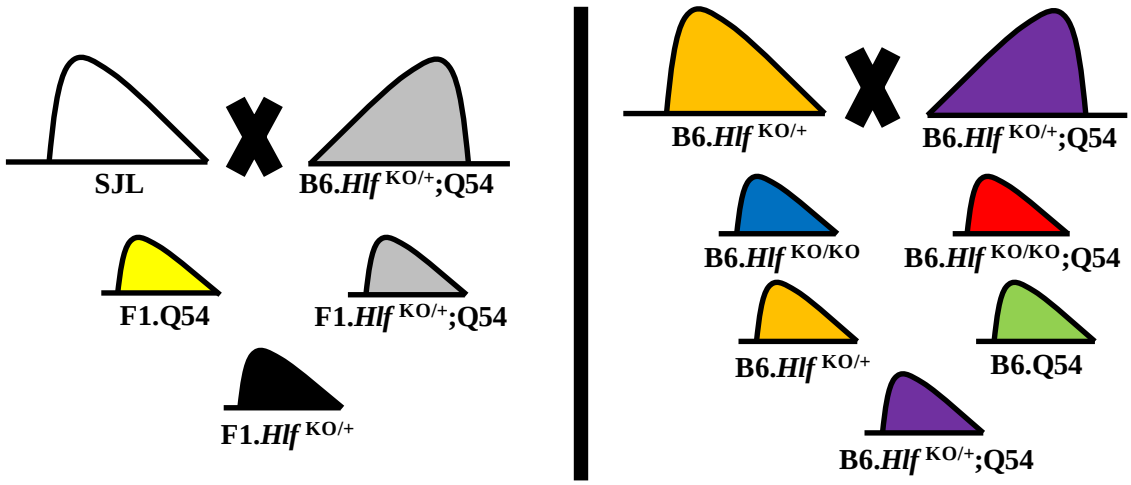

Figure 3.7 Generation of *Hlf* X B6.Q54 Double Mutant Mice
Two background strains of *Hlf* double mutant mice were generated. To produce animals for phenotyping experiments, B6.*Hlf* $^{KO/+}$;Q54 males were crossed with either B6.*Hlf* $^{KO/+}$ or SJL females. Crossing B6.*Hlf* $^{KO/+}$;Q54 with B6.*Hlf* $^{KO/+}$ resulted in homozygous B6.*Hlf* $^{KO/KO}$;Q54 and heterozygous B6.*Hlf* $^{KO/+}$;Q54 test mice and control littermates. Crossing B6.*Hlf* $^{KO/+}$;Q54 with SJL resulted in F1.*Hlf* $^{KO/+}$;Q54 test mice and control offspring.

Table 3.2 Ratio of Genotypes Observed From B6.*Hlf* $^{KO/+}$;Q54 X B6.*Hlf* $^{KO/+}$
B6.*Hlf* $^{KO/+}$;Q54 males were crossed with B6.*Hlf* $^{KO/+}$ females to generate homozygous B6.*Hlf* $^{KO/KO}$;Q54 and heterozygous B6.*Hlf* $^{KO/+}$;Q54 mice and control littermates. Males were born at the expected Mendelian ratios, while females differed significantly from the expected genotype ratios, predominantly homozygous knockout B6.*Hlf* $^{KO/KO}$;Q54, heterozygous knockout B6.*Hlf* $^{KO/+}$;Q54 and B6.*Hlf* $^{KO/+}$.

	Genotype	# Observed	# Expected	Chi Sq
Males (n=392)	B6	56	49	$\chi^2 = 2.571$ $p > 0.766$
	B6.Q54	48	49	
	B6.*Hlf* $^{KO/+}$	94	98	
	B6.*Hlf* $^{KO/+}$;Q54	104	98	
	B6.*Hlf* $^{KO/KO}$	42	49	
	B6.*Hlf* $^{KO/KO}$;Q54	48	49	
Females (n=414)	B6	55	52	$\chi^2 = 17.836$ $p < 0.003$
	B6.Q54	48	52	
	B6.*Hlf* $^{KO/+}$	86	104	
	B6.*Hlf* $^{KO/+}$;Q54	129	104	
	B6.*Hlf* $^{KO/KO}$	62	52	
	B6.*Hlf* $^{KO/KO}$;Q54	34	52	

Effect of Hlf on F1.Q54 Phenotype

Offspring from the B6.*Hlf* $^{KO/+}$ by SJL cross underwent 30 minute video-taped observations for visible, spontaneous seizures at three and six weeks of age. The two time points were then combined to determine the seizure frequency in 60 minutes. Seizure analysis of F1.*Hlf* $^{KO/+}$ offspring (Male n=15; Female n=15) revealed no apparent motor seizures. This was consistent with previous work on *Hlf* knockout mice, not in combination with other PAR bZIP knockout lines, which reported no evidence of seizures or neurological abnormalities (19). Therefore, average seizure frequency was compared by Student's t-test between double mutant F1.*Hlf* $^{KO/+}$;Q54 and F1.Q54 controls.

No significant difference in seizure frequency was observed between F1.*Hlf* $^{KO/+}$;Q54 and F1.Q54 controls in either males ($p > 0.348$) or females ($p > 0.124$) (Figures 3.8 and 3.9). Although it was not significant, there was a trend towards increased seizure frequency in F1.*Hlf* $^{KO/+}$;Q54 females compared to F1.Q54 controls.

Following seizure phenotyping, mice from the F1 cross were retained until 12 weeks of age to determine if heterozygous loss of *Hlf* altered the lifespan of F1.Q54 mice. We did not observe any significant effect on the survival of F1.*Hlf* $^{KO/+}$;Q54 mice compared to F1.Q54 controls (Data not shown).

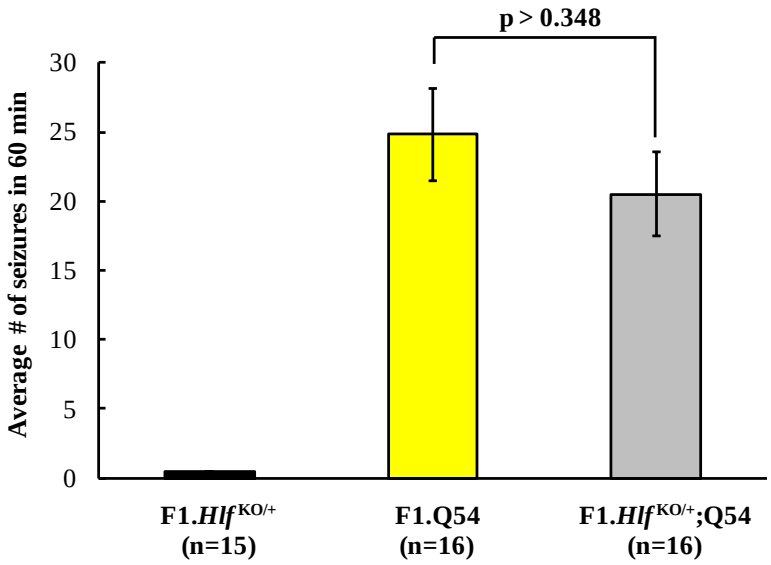

Figure 3.8 Average Seizure Frequency of B6.*Hlf* $^{KO/+}$;Q54 X SJL Males
Average number of seizures in 60 minutes for each genotype is shown. Average seizure frequencies for males are as follows: F1.*Hlf* $^{KO/+}$= 0, F1.Q54= 24.8 ± 3.36 and F1.*Hlf* $^{KO/+}$;Q54= 20.5 ± 3.03. P-value was determined by Student's t-test. Error bars represent SEM.

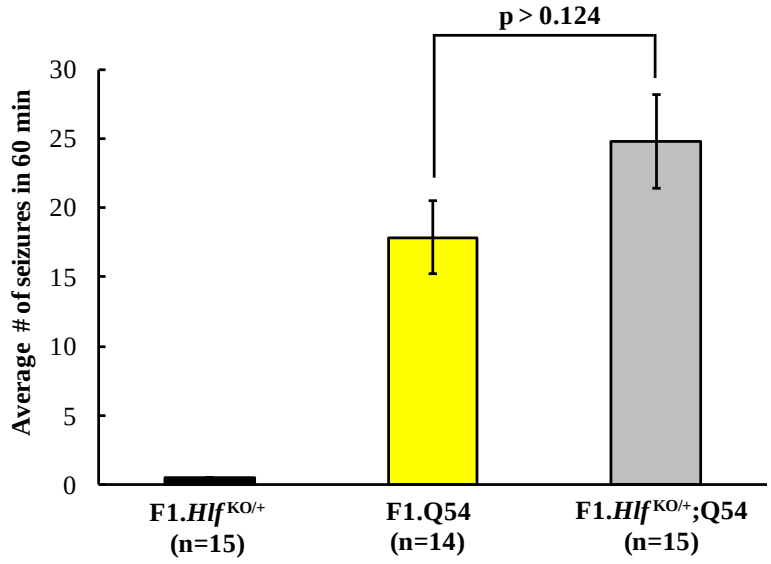

Figure 3.9 Average Seizure Frequency of B6.*Hlf* $^{KO/+}$;Q54 X SJL Females
Average number of seizures in 60 minutes for each genotype is shown. Average seizure frequencies for females are as follows: F1.*Hlf* $^{KO/+}$= 0, F1.Q54= 17.9 ± 2.63 and F1.*Hlf* $^{KO/+}$;Q54= 24.8 ± 3.34. P-value was determined by Student's t-test. Error bars represent SEM.

Effect of Hlf on B6.Q54 Phenotype

Offspring from the B6.*Hlf* $^{KO/+}$;Q54 by B6.*Hlf* $^{KO/+}$ cross underwent 30 minute video-taped observations as described. Seizure analysis of B6.*Hlf* $^{KO/+}$ *(*Male n=15; Female n=15) and B6.*Hlf* $^{KO/KO}$ (Male n=15; Female n=15) offspring revealed no motor seizures, therefore average seizure frequency was compared between heterozygous knockout B6.*Hlf* $^{KO/+}$;Q54, homozygous knockout B6.*Hlf* $^{KO/KO}$;Q54 mice and B6.Q54 controls by ANOVA.

A significant difference in seizure frequency was observed in males ($F_{(2,38)}$= 6.348, $p < 0.004$). Double mutant heterozygous B6.*Hlf* $^{KO/+}$;Q54 and homozygous B6.*Hlf* $^{KO/KO}$;Q54 males had significantly ($p < 0.001$ and $p < 0.023$, respectively) lower seizure frequencies than B6.Q54 controls (Figure 3.10). However, there was no significant difference in seizure frequency between heterozygous knockout B6.*Hlf* $^{KO/+}$;Q54 and homozygous knockout B6.*Hlf* $^{KO/KO}$;Q54 male mice ($p > 0.353$). Although we originally hypothesized that loss of *Hlf* would increase seizure frequency, a suggested cause for the reduced seizure frequency emerged from detailed analysis of the video records. Male homozygous B6.*Hlf* $^{KO/KO}$;Q54 mice experienced a significant number of GTCS (χ^2= 6.209, $p < 0.045$) (Figure 3.11). This more severe phenotype is not observed in Q54 mice in this age range (5, 7, 8, 32). No significant difference in seizure frequency was observed in females ($F_{(2,36)} = 0.744$, $p > 0.482$) (Figure 3.12).

Following seizure phenotyping, we retained mice until 12 weeks of age to determine if loss of *Hlf* altered the lifespan of B6.Q54 mice. We did not observe any effect on the survival of heterozygous B6.*Hlf* $^{KO/+}$;Q54 or homozygous B6.*Hlf* $^{KO/KO}$;Q54 mice compared to B6.Q54 controls in either males or females (Data not shown).

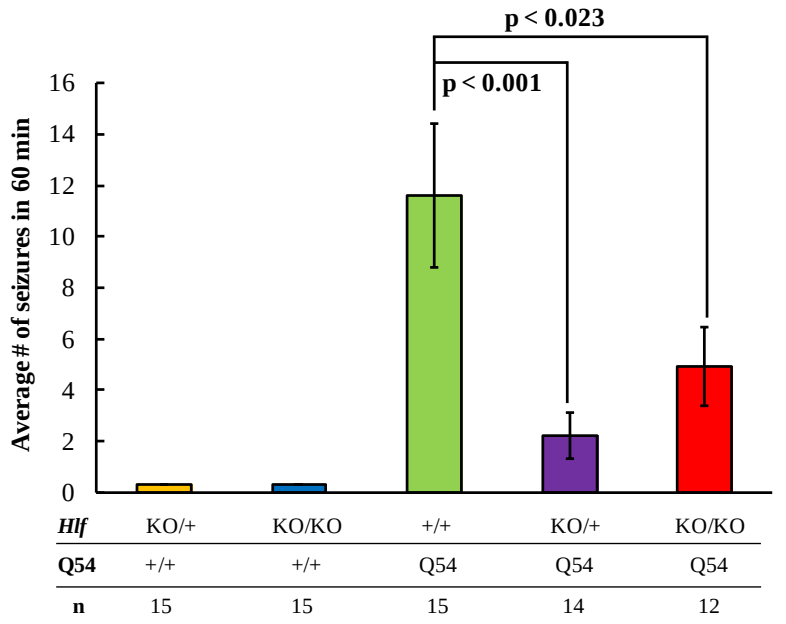

Figure 3.10 Average Seizure Frequency of B6.*Hlf* $^{KO/+}$;Q54 X B6.*Hlf* $^{KO/+}$ Males
Average number of seizures in 60 minutes for each genotype is shown. Average seizure frequencies for males are as follows: B6.*Hlf* $^{KO/+}$= 0, B6.*Hlf* $^{KO/KO}$= 0, B6.Q54= 11.6 ± 2.8, B6.*Hlf* $^{KO/+}$;Q54= 2.21 ± 0.89 and B6.*Hlf* $^{KO/KO}$;Q54= 4.92 ± 1.52. Significant differences between groups were determined by ANOVA: $F_{(2,38)} = 6.348$, $p < 0.004$. P-values determined by Fishers PLSD *post hoc* test. Error bars represent SEM.

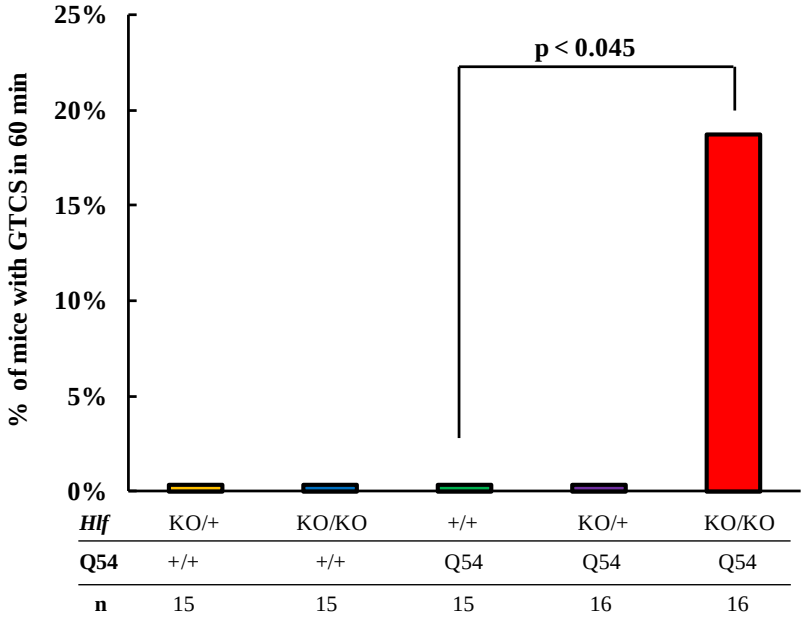

Figure 3.11 Percentage of B6.*Hlf* $^{KO/+}$;Q54 X B6.*Hlf* $^{KO/+}$ Males Experiencing GTCS
Percentage of mice experiencing GTCS in 60 minutes for each genotype is shown. Percentages for males are as follows: B6.*Hlf* $^{KO/+}$= 0%, B6.*Hlf* $^{KO/KO}$= 0%, B6.Q54= 0%, B6.*Hlf* $^{KO/+}$;Q54= 0% and B6.*Hlf* $^{KO/KO}$;Q54= 18.9%. P-value was determined by Chi-squared analysis ($\chi^2 = 6.209$, $p < 0.045$).

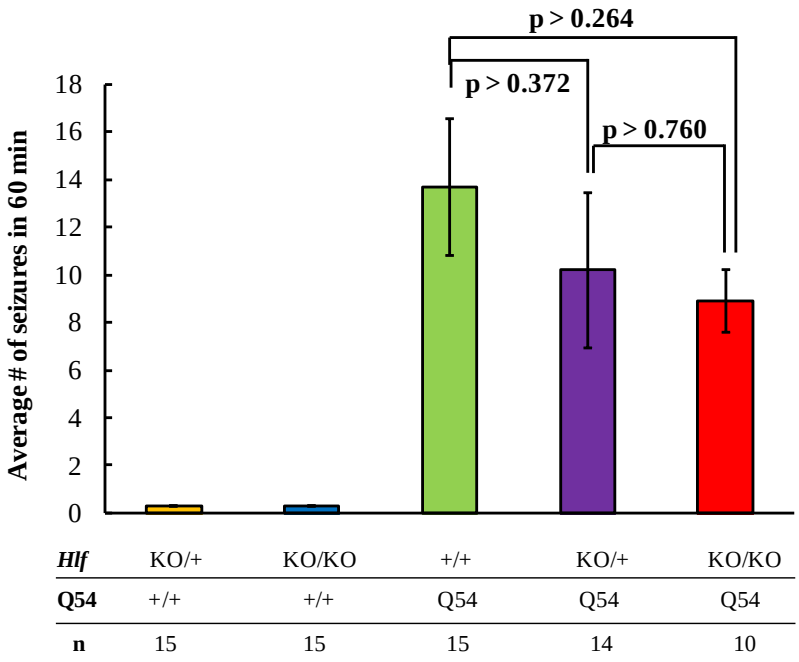

Figure 3.12 Average Seizure Frequency of B6.*Hlf* $^{KO/+}$;Q54 X B6.*Hlf* $^{KO/+}$ Females
Average number of seizures in 60 minutes for each genotype is shown. Average seizure frequencies for females are as follows: B6.*Hlf* $^{KO/+}$= 0, B6.*Hlf* $^{KO/KO}$= 0, B6.Q54= 13.7 ± 2.87, B6.*Hlf* $^{KO/+}$;Q54= 10.2 ± 3.25 and B6.*Hlf* $^{KO/KO}$;Q54= 8.9 ± 1.29. Significant differences between groups were determined by ANOVA: $F_{(2,36)}$ = 0.744, p > 0.482. P-values determined by Fishers PLSD *post hoc* test. Error bars represent SEM.

Effect of Pyridoxine Deficient and Enriched Diet on Q54

To further evaluate our *Hlf* hypothesis, we tested the effects of pyridoxine deficient and enriched diets on the Q54 phenotype. Deletion of the PAR bZIP transcription factor family (*Hlf, Dbp* and *Tef*) in a knock-out mouse model resulted in mice with spontaneous, generalized tonic-clonic and absence seizures (19). Microarray expression analysis was utilized to determine contributors to the epilepsy phenotype (19). A two-fold reduction in PDXK, a key enzyme involved in the conversion of pyridoxine to PLP, was discovered (19). PLP is a key coenzyme involved in amino acid and neurotransmitter metabolism, including GABA (Figure 3.13). We hypothesized that B6.Q54 and F1.Q54 mice on pyridoxine deficient diet would have increased seizure frequency compared to controls, due to reduced PLP levels.

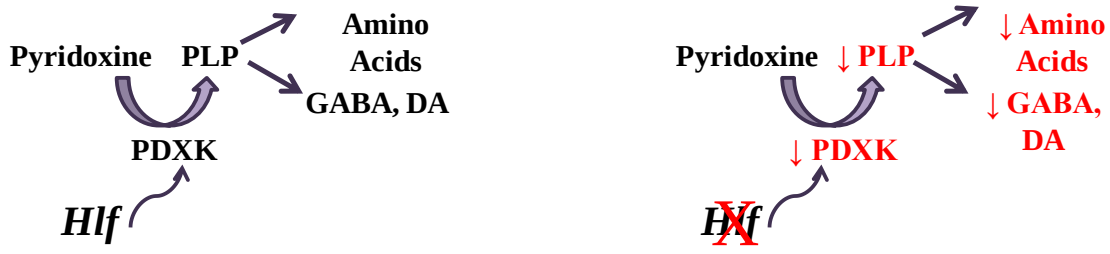

Figure 3.13 Simplified Pyridoxine Pathway
Loss of the PAR bZIP transcription factor family (*Hlf*, *Dbp* and *Tef*) result in mice with spontaneous GTCS and a two-fold reduction in PDXK levels. We predict loss of *Hlf* alters the pyridoxine pathway, consequently reducing GABA and neurotransmitter levels.

Furthermore, we evaluated the effect of a pyridoxine enriched diet, to determine if elevated levels of pyridoxine reduced seizure frequency in B6.Q54 and F1.Q54 mice. Male and female B6.Q54 and F1.Q54 mice were placed on either the modified or control diet at three weeks of age and were maintained on the diet for six weeks. At nine weeks of age, all mice underwent 30 minute video-taped observations as described above, to assess seizure frequency. We also monitored survival until nine weeks of age to determine if pyridoxine deficient or enriched diets had any effect on the lifespan. Although we provided modified and control diets to B6, F1, B6.*Hlf* $^{KO/+}$ and B6.*Hlf* $^{KO/KO}$ male and female mice, no diet effects were detected in any of these groups. Therefore data for these groups are not shown.

No significant difference in seizure frequency was observed in B6.Q54 males maintained on the modified diets compared to control diet ($p > 0.175$, $p > 0.598$) (Figure 3.14). While not statistically significant, there was a trend toward increased seizure frequency in F1.Q54 males maintained on the deficient and enriched diets compared to control diet ($p > 0.055$ and $p > 0.065$, respectively).

No significant effect was observed when comparing the survival of B6.Q54 males maintained on either modified diet compared to controls (Data not shown). However, F1.Q54 males maintained on the enriched diet had a significant reduction in lifespan compared to controls ($p < 0.017$) (Figure 3.15). The seizure and survival data indicate that the F1.Q54 male phenotype is altered by both pyridoxine deficient and enriched diets, while the B6.Q54 male phenotype is not changed by either modified diet.

A significant difference in seizure frequency was observed in females. B6.Q54 females maintained on a pyridoxine deficient diet had significantly more ($p < 0.017$) seizures compared to B6.Q54 females on the control diet (Figure 3.16). No change was observed in B6.Q54 females on the pyridoxine enriched diet ($p > 0.893$). No significant difference in seizure frequency was observed in F1.Q54 females maintained on either modified diet ($p > 0.451$ and $p > 0.564$, respectively) (Figure 3.16).

A significant reduction in the lifespan of B6.Q54 females maintained on the pyridoxine deficient diet compared to controls was identified ($p < 0.011$) (Figure 3.17). No significant effect was observed when comparing the survival of F1.Q54 females maintained on either modified to controls (Data not shown). The seizure and survival data indicate that the B6.Q54 female phenotype is altered by a pyridoxine deficient diet, while the F1.Q54 female phenotype is not changed by either modified pyridoxine diet.

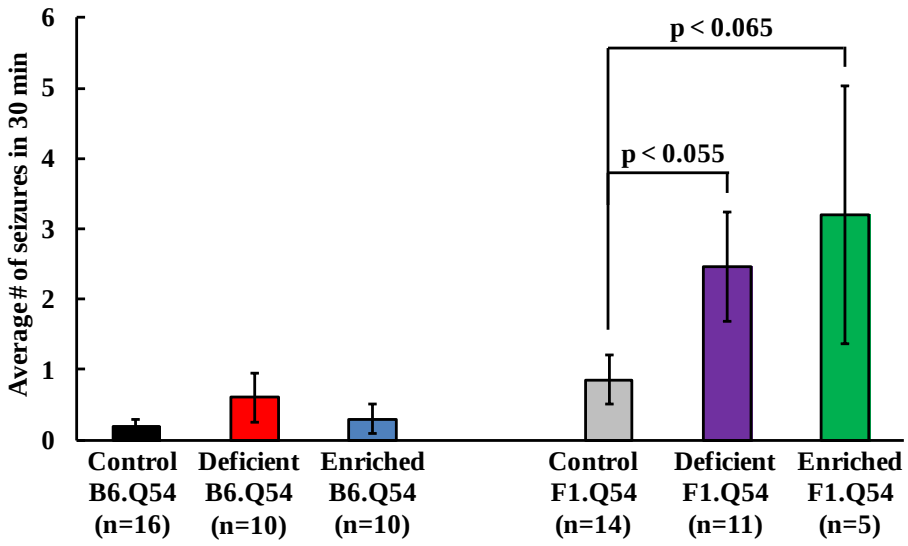

Figure 3.14 Average Seizure Frequency of Males on Modified Pyridoxine Diets
Average number of seizures in 30 minutes for each genotype is shown. Average seizure frequencies for males are as follows: Control B6.Q54= 0.188 ± 0.101, Deficient B6.Q54= 0.6 ± 0.34, Enriched B6.Q54= 0.3 ± 0.213, Control F1.Q54= 0.587 ± 0.345, Deficient F1.Q54= 2.46 ± 0.779 and Enriched F1.Q54= 3.2 ± 1.828. Significant differences were determined by Student's t-test. Error bars represent SEM.

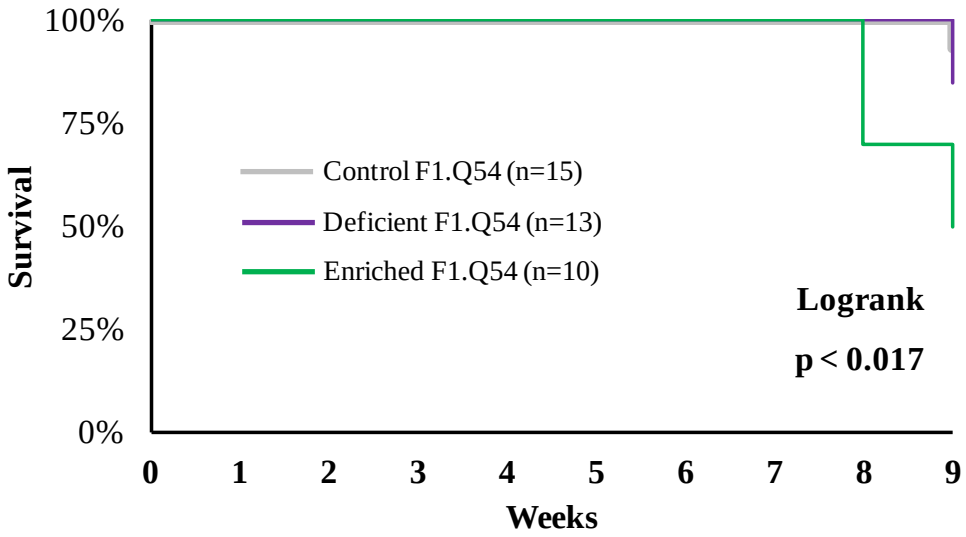

Figure 3.15 Kaplan Meier Survival of F1.Q54 Males on Modified Pyridoxine Diets
Survival of F1.Q54 male mice maintained on control, deficient or enriched pyridoxine diets are shown. Survival was monitored until 9 weeks of age. P-value was determined by Logrank Mantel-Cox.

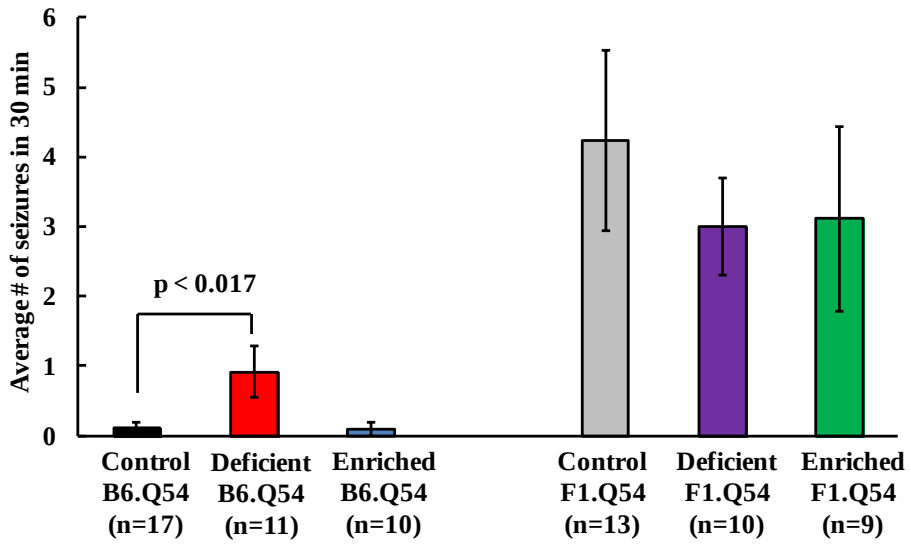

Figure 3.16 Average Seizure Frequency of Females on Modified Pyridoxine Diets
Average number of seizures in 30 minutes for each genotype is shown. Average seizure frequencies for females are as follows: Control B6.Q54= 0.117 ± 0.081, Deficient B6.Q54= 0.91 ± 0.368, Enriched B6.Q54= 0.1 ± 0.1, Control F1.Q54= 4.23 ± 1.29, Deficient F1.Q54= 3 ± 0.699 and Enriched F1.Q54= 3.11 ± 1.328. Significant differences were determined by Student's t-test. Error bars represent SEM.

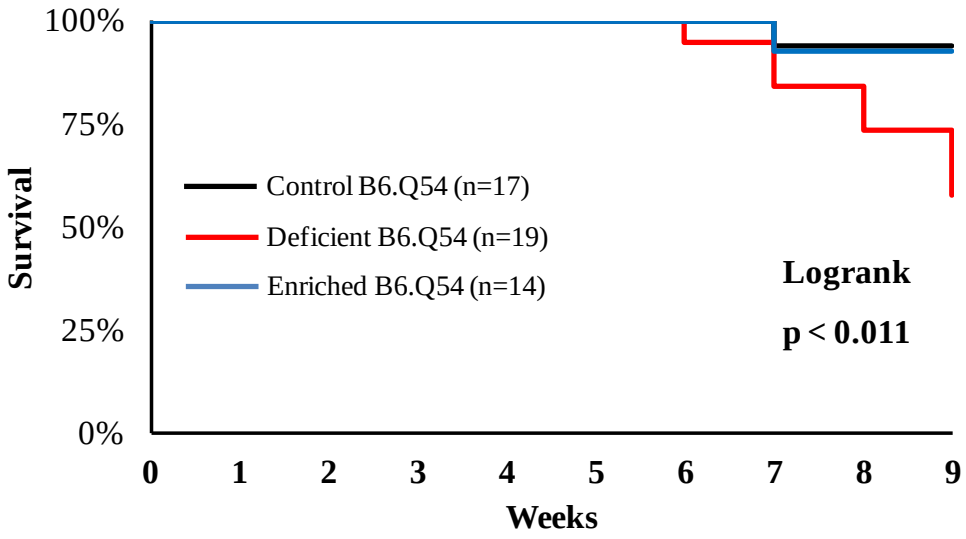

Figure 3.17 Kaplan Meier Survival of B6.Q54 Females on Modified Pyridoxine Diets
Survival of B6.Q54 female mice maintained on control, deficient or enriched pyridoxine diets are shown. Survival was monitored until 9 weeks of age. P-value was determined by Logrank Mantel-Cox.

Effect of Pyridoxine Deficient Diet on Neurochemistry

A subset of mice maintained on the pyridoxine deficient and control diets were used for neurochemistry studies. Neurochemistry assays for biogenic amine neurotransmitters and metabolites, amino acids and amino acid neurotransmitters were used to determine if the deficient diet altered brain chemistry.

Relevant to our phenotyping studies, F1.Q54 males on the deficient diet had a significant increase in glutamate and GABA concentration compared to controls (Figure 3.18). This supports our seizure data which indicated F1.Q54 males on the deficient diet had more seizures compared to controls (Figure 3.14). Although B6.Q54 females experienced increased seizures on the deficient diet, no significant difference in glutamate or GABA was identified (Data not shown).

Regardless of genotype, all males maintained on the pyridoxine deficient diet had detectable brain concentrations of proline, while controls did not (Figure 3.19). B6 and B6.Q54 males on the deficient diet had significant differences in proline concentration compared to controls ($p < 0.027$ and $p < 0.024$, respectively). Proline was not detected in any females. Altered proline concentrations, such as those in Hyperprolinemia type II, cause pyridoxine deficiency by endogenous accumulation of the PLP deactivator P5C, resulting in pyridoxine dependent seizures (21, 33). This suggests that males, in particular B6 and B6.Q54, are more susceptible to the metabolic changes associated with a pyridoxine deficient diet, although an obvious phenotype was not observed.

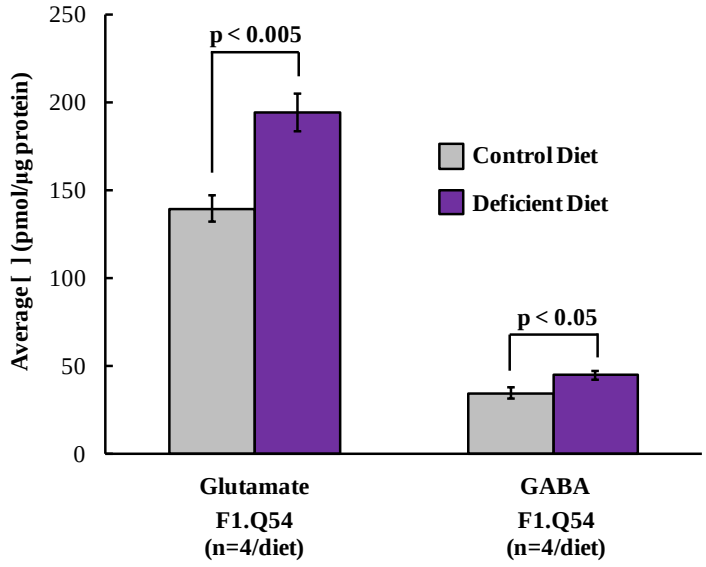

Figure 3.18 F1.Q54 Male Brain Concentrations of Glutamate and GABA

F1.Q54 male mice were maintained on the deficient or control diet for six weeks. Glutamate and GABA concentrations were assayed in forebrain. Average concentrations are as follows: Glutamate: F1.Q54 control= 139.6 ± 7.34 and F1.Q54 deficient= 194.4 ± 10.7. GABA: F1.Q54 control= 34.4 ± 3.11 and F1.Q54 deficient= 44.5 ± 2.71. P-values determined by Student's t-test. Error bars represent SEM.

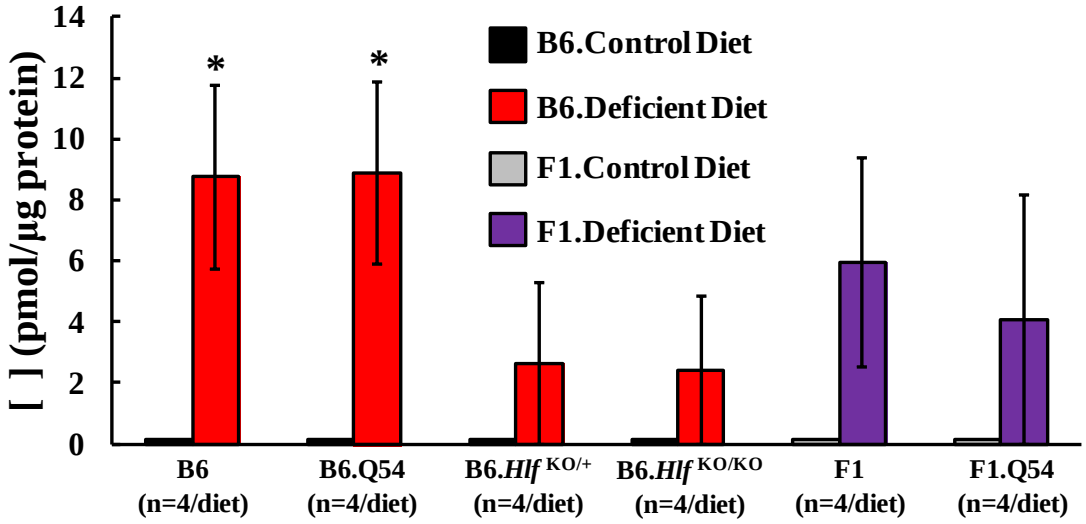

Figure 3.19 Proline Concentrations Identified in Males

Male mice were maintained on the deficient or control diet for six weeks. Proline concentrations were assayed in forebrain. Average concentrations are as follows: B6, B6.Q54, B6.*Hlf* $^{KO/+}$, B6.*Hlf* $^{KO/KO}$, F1 and F1.Q54 controls= 0, B6 deficient= 8.74 ± 3.02, B6.Q54 deficient= 8.89 ± 2.97, B6.*Hlf* $^{KO/+}$ deficient= 2.63 ± 2.63, B6.*Hlf* $^{KO/KO}$ deficient= 2.42 ± 2.42, F1 deficient= 5.96 ± 3.45 and F1.Q54 deficient= 4.07 ± 4.07. P-values determined by Student's t-test. * represents significance compared to control diet. * < 0.01. Error bars represent SEM.

Effect of Hlf on the Dravet Phenotype of Scn1a$^{KO/+}$ Heterozygous Knockout Mice

To determine whether *Hlf* would modify other types of epilepsy, we assessed the ability of *Hlf* to alter the Dravet phenotype of *Scn1a*$^{KO/+}$ heterozygous knockout mice. To do this, we generated double mutant mice by crossing B6.*Hlf* $^{KO/+}$ females with 129.*Scn1a*$^{KO/+}$ males to generate F1 double heterozygous knockout F1.*Hlf* $^{KO/+}$;*Scn1a*$^{KO/+}$ offspring and controls carrying either F1.*Hlf* $^{KO/+}$ or F1.*Scn1a*$^{KO/+}$. Double heterozygous and control mice were then monitored for survival until 12 weeks of age to determine if loss of *Hlf* altered the lifespan of F1.*Scn1a*$^{KO/+}$ mice. A significant reduction in lifespan was observed between F1.*Hlf* $^{KO/+}$;*Scn1a*$^{KO/+}$ and control F1.*Scn1a*$^{KO/+}$ mice ($p < 0.001$) (Figure 3.20). By four weeks of age, only 45% of double heterozygous knockout F1.*Hlf* $^{KO/+}$;*Scn1a*$^{KO/+}$ mice survived, compared to the 71% survival rate of F1.*Scn1a*$^{KO/+}$ mice. By 12 weeks of age, only 29% of F1.*Hlf* $^{KO/+}$;*Scn1a*$^{KO/+}$ mice survived compared to 42% of F1.*Scn1a*$^{KO/+}$ mice. As expected, F1.*Hlf* $^{KO/+}$ mice have no phenotype with 100% survival throughout the study.

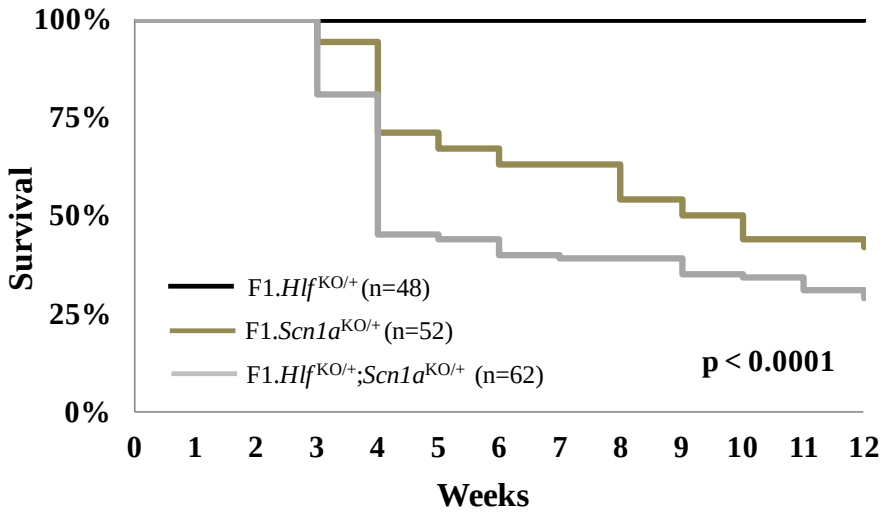

Figure 3.20 Kaplan Meier Survival of B6.*Hlf* $^{KO/+}$ X 129.*Scn1a*$^{KO/+}$ Mice
Survival of F1.*Hlf* $^{KO/+}$, F1.*Scn1a*$^{KO/+}$ and F1.*Hlf* $^{KO/+}$;*Scn1a*$^{KO/+}$ mice are shown. Survival was monitored until 12 weeks of age. Kaplan Meier analysis was performed. P-value determined by Logrank Mantel-Cox.

Discussion

We previously mapped a dominant locus on chromosome 11, designated *Moe1,* which modified seizure susceptibility of Q54 mice. Fine-mapping and RNA-Seq data suggested two sex-specific candidate modifier genes at the *Moe1* locus: *Cacna1g* in females and *Hlf* in males (8). To determine the contribution of these genes to the seizure phenotype of Q54 mice, we utilized *Cacna1g* BAC transgenic and *Hlf* targeted knockout mouse models. We systematically evaluated the candidate modifier genes by testing their ability to enhance or reduce seizure severity of the Q54 phenotype in double mutant mice. In addition to seizure analysis, we also evaluated our *Hlf* hypothesis by testing the effects of modified dietary pyridoxine on the Q54 phenotype and assessed *Hlf's* ability to modify the Dravet phenotype of $Scn1a^{KO/+}$ mice.

Validation of Cacna1g as a Genetic Modifier of F1.Q54 Mice

Cacna1g, encoding the T-type calcium channel α_1 subunit, was prioritized as a top candidate modifier gene of the *Moe1* locus based on previous research attributing *Cacna1g* mutations to human and mouse epilepsy (8, 10, 11). *Cacna1g* was hypothesized to modify the Q54 seizure phenotype through significant strain-specific differences in expression profiles of transcripts which have functional diversity (8). *Cacna1g* channels preferentially expressed in SJL females reduce synchronous thalamocortical oscillations, a common feature of epilepsy, while B6 females express *Cacna1g* channels prone to hyperexcitability (8, 16-18). Therefore, we wanted to systematically evaluate overexpression of B6 *Cacna1g* to determine if it increased the seizure frequency of F1.Q54 mice.

To evaluate the contribution of *Cacna1g* to the seizure phenotype of F1.Q54 mice, we generated a *Cacna1g* BAC transgenic mouse model. It was determined that the 1G5 line had X-chromosome integration of an estimated 5-8 transgene copies and expressed *Cacna1g* at increased levels compared to wildtype. Double transgenic F1.1G5;Q54 males had significantly more seizures than F1.Q54 controls, while there was no difference in females.

Although previous mapping data (Chapter II) suggested *Cacna1g* as a female-specific modifier, results from female F1.1G5;Q54 mice were difficult to interpret due to the possibility that the transgene may be subject to X-inactivation (8). Prior work indicated transgene integration onto the X chromosome can result in partial or complete inactivation (8, 34-36). Total or partial inactivation of the 1G5 BAC may have resulted in incomplete transgene expression. Consequently, seizure frequency of F1.1G5:Q54 females may not be altered due to technical reasons. Moreover, it is possible that females, which already have a high seizure frequency, may experience a ceiling effect.

While our previous mapping data did not suggest *Cacna1g* as a candidate modifier in males, it is not unexpected as *CACNA1G* is directly involved in human epilepsy (8, 11). We hypothesize that males may have a protective modifier gene in *Moe1* which may have masked *Cacna1g's* effect on seizure frequency in our mapping study (8). Future work is necessary to verify the full modifier potential of *Cacna1g*, including generation of additional *Cacna1g* BAC transgenic lines.

Validation of Hlf as a Genetic Modifier of Q54 Mice

Hlf, encoding hepatic leukemia factor, a PAR bZIP transcription factor, was prioritized as a top candidate modifier gene of the *Moe1* locus in males. This was based on a PAR bZIP transcription factor family knockout mouse model that experienced spontaneous GTCS and absence seizures (19). Further analysis of the model revealed a reduction in PDXK, a key enzyme involved in the conversion of pyridoxine to PLP (19). PLP is a key coenzyme involved in amino acid and neurotransmitter metabolism, including GABA and glutamate (20). PLP has a substantial connection to human and mouse epilepsy, as pyridoxine-dependent epilepsies are successfully treated with PLP and mice defective in PLP metabolism experience fatal seizures (21, 25).

Hlf was hypothesized to modify the Q54 seizure phenotype by contributing to strain-specific PDXK and PLP levels (Figure 3.1) (8). *Hlf* is expressed at a significantly reduced level in B6 males compared to SJL (8). Furthermore, we observed a trend toward significant reduction of PDXK expression ($p > 0.135$) in B6 males compared to SJL in brain RNA-Seq analysis (Unpublished observations, N. Hawkins and J. Kearney,). These reductions in B6 may decrease PLP levels, consequently increasing seizure susceptibility. Therefore we wanted to systematically evaluate if loss of *Hlf* would increase seizure frequency of F1.Q54 and B6.Q54 mice.

On the F1 strain, no significant differences in seizure frequency or survival were observed between heterozygous knockout F1.*Hlf* $^{KO/+}$;Q54 mice and F1.Q54 controls in either sex. This outcome was expected as SJL mice express *Hlf* at significantly increased levels compared to B6. The single SJL *Hlf* copy may be enough to support sufficient PDXK and PLP levels.

On the B6 strain, double mutant heterozygous knockout B6.*Hlf* $^{KO/+}$;Q54 and homozygous knockout B6.*Hlf* $^{KO/KO}$;Q54 males had significantly lower seizure frequencies compared to B6.Q54 controls. We originally hypothesized that loss of *Hlf* would increase seizure frequency. While B6.*Hlf* $^{KO/+}$;Q54 and B6.*Hlf* $^{KO/KO}$;Q54 had reduced total focal motor seizures compared to controls, B6.*Hlf* $^{KO/KO}$;Q54 males experienced GTCS not traditionally seen in the B6.Q54 model. Although much older B6.Q54 mice may experience rare GTCS, in our previous studies of B6.Q54 we never observed GTCS at three or six weeks of age (5, 7, 8, 32). Therefore, we conclude that complete loss of *Hlf* exacerbated the seizure phenotype of B6.Q54 male mice.

No significant difference in seizure frequency was observed in B6.*Hlf* $^{KO/+}$;Q54 or B6.*Hlf* $^{KO/KO}$;Q54 females. Although an overt seizure phenotype was not detected in either heterozygous or homozygous knockout females, B6.*Hlf* $^{KO/+}$, heterozygous B6.*Hlf* $^{KO/+}$;Q54 and homozygous B6.*Hlf* $^{KO/KO}$;Q54 females were born at significantly altered ratios than expected. The ratio shift suggests reduced viability of B6.*Hlf* $^{KO/KO}$;Q54 females. Future studies will determine if B6.*Hlf* $^{KO/KO}$;Q54 females are dying before tail biopsy at P14 or are not being born. Either outcome suggests *Hlf* modifies the survival of female B6.Q54 mice.

In addition to seizure analysis, we evaluated our *Hlf* hypothesis by testing the effects of pyridoxine deficient and enriched diets on the Q54 phenotype. We hypothesized that a pyridoxine deficient diet would increase seizure frequency, based on past data which revealed mice maintained on a deficient diet experienced increased seizure frequency (22-24). B6.Q54 females on the pyridoxine deficient diet had increased seizure frequency and reduced survival compared to controls. The B6.Q54 male seizure

phenotype was not altered. This could be due to a variety of reasons, including the relatively short length of time mice were maintained on the deficient diet. Neurochemistry studies revealed B6.Q54 males maintained on the deficient diet had significant levels of proline detected in their brains, whereas controls had zero. Hyperprolinemia has been associated with seizures and epilepsy (21). This may indicate the start of an altered metabolic state in male B6.Q54 mice and suggests that a phenotype may emerge if the deficient diet was continued for longer than six weeks. Although F1.Q54 females maintained on the pyridoxine deficient diet had no changes in seizure frequency or survival, F1.Q54 males on the deficient diet had near significant increases in seizure frequency. Neurochemistry studies revealed F1.Q54 males had significant changes in glutamate and GABA concentrations compared to controls. Given the functions of glutamate and GABA, we predict these altered levels may contribute to the modified seizure phenotype of F1.Q54 male mice.

We hypothesized a pyridoxine enriched diet would decrease seizure frequency, given pyridoxine dependent epilepsies are successfully treated with PLP (21). No reduction in seizure frequency was observed in B6.Q54 males or females maintained on the pyridoxine enriched diet. It is possible that the already low seizure frequency of B6.Q54 mice may make significant reductions impossible due to floor effects. Alternatively, pyridoxine enrichment may not have an effect on seizure frequency in B6.Q54 mice. F1.Q54 females maintained on the pyridoxine enriched diet had no changes in seizure frequency or survival. F1.Q54 males had increased seizure frequency and reduced survival on the enriched diet compared to controls. Of the ten F1.Q54 males maintained on the enriched diet, only five survived until 9 weeks of age. At least in

males, pyridoxine enrichment has no positive effect on reducing the seizure phenotype of F1.Q54 mice, rather it exacerbates the seizure phenotype.

Finally, *Hlf* was shown to modify the Dravet phenotype of *Scn1a*$^{KO/+}$ heterozygous knockout mice. Double heterozygous knockout F1.*Hlf* $^{KO/+}$;*Scn1a*$^{KO/+}$ mice had a significant reduction in lifespan compared to controls. By four weeks of age, only 45% of F1.*Hlf* $^{KO/+}$;*Scn1a*$^{KO/+}$ mice survived compared to 71% of F1.*Scn1a*$^{KO/+}$ controls. The *Scn1a*$^{KO/+}$ mice recapitulate many features of Dravet syndrome, including development of early onset epilepsy, spontaneous seizures and premature death (37, 38). It is hypothesized that reduced sodium currents in GABAergic inhibitory interneurons are responsible for the *Scn1a*$^{KO/+}$ seizure phenotype (38). Our *Scn1a*$^{KO/+}$ model replicated those studies and identified an increase in sodium current density in pyramidal neurons (28). The loss of *Hlf,* hypothesized to reduce PDXK and PLP, may alter neurotransmitter synthesis. This in combination with *Scn1a*$^{KO/+}$ increased hyperexcitability, may be responsible for the decreased survival seen in F1.*Hlf* $^{KO/+}$;*Scn1a*$^{KO/+}$ mice.

Through seizure analysis and modified diet studies, *Hlf* was shown to be a modifier of the Q54 phenotype. Although previous data did not suggest *Hlf* as a female candidate modifier gene, it is not unexpected, as loss of *Hlf* is hypothesized to alter PLP enzyme levels and thus increase seizure susceptibility. Furthermore, *Hlf* was shown to modify the phenotype of Dravet mice. Future identification of *HLF* risk variants in epilepsy patients would validate this gene as a contributor to human epilepsy.

Verification of *Cacna1g* and *Hlf* as epilepsy modifier genes may suggest new targets for improved treatment of epilepsy and advance molecular diagnostic capabilities by identifying those patients who are most at-risk for developing severe epilepsy.

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CHAPTER IV

NEURONAL VOLTAGE-GATED ION CHANNELS ARE GENETIC MODIFIERS OF GENERALIZED EPILEPSY WITH FEBRILE SEIZURE PLUS*

Introduction

During the past 20 years, research has revealed several genes underlying rare monogenic forms of idiopathic generalized epilepsy (IGE); however there has been less progress towards the identification of genes involved in the more common, genetically complex forms of IGE (1-3). Many of the genes now known to cause monogenic forms of epilepsy encode neuronal ion channel subunits, including voltage-gated sodium and potassium channels. Mutations in the voltage-gated sodium channel genes *SCN1A*, *SCN2A* and *SCN1B* result in genetic epilepsy with febrile seizures plus (GEFS+) (4-6).We recently generated a mouse model of GEFS+ by introducing the human *SCN1A*^{R1648H} GEFS+ mutation, which was identified in a large pedigree with 13 affected members, into the orthologous mouse *Scn1a* gene (7).

Scn1a$^{R1648H/+}$ heterozygous mutants display a normal lifespan, reduced thresholds to flurothyl and hyperthermia induced seizures and infrequent spontaneous generalized seizures as adults (7). *Scn1a*$^{R1648H/R1648H}$ homozygous mice exhibit spontaneous generalized seizures and have an average lifespan of 18.5 days. Cortical interneurons from *Scn1a*$^{R1648H/+}$ and *Scn1a*$^{R1648H/R1648H}$ mice display slowed recovery from inactivation, increased use-dependence and a reduced ability to fire action potentials.

Modified from*: Hawkins NA, Martin MS, Frankel WN, Kearney JA and Escayg A. (2011) Neuronal voltage-gated ion channels are genetic modifiers of generalized epilepsy with febrile seizure plus. *Neurobiology of Disease*, **41, 655-660.

These electrophysiological abnormalities are predicted to reduce the level of GABAergic inhibition, providing a mechanism for seizure generation (7).

Mutations in the voltage-gated sodium channel gene *SCN2A* have also been associated with human epilepsy syndromes including GEFS+, benign familial neonatal-infantile seizures (BFNIS), Dravet and Ohtahara syndromes (8-10). The transgenic mouse model $Scn2a^{Q54}$ (Q54) has a gain-of function mutation in *Scn2a,* resulting in a progressive epilepsy phenotype characterized by focal motor seizures that begin in the second month of life. Later development of secondary generalized seizures and a reduced lifespan also occur. Hippocampal pyramidal neurons from Q54 mice exhibit increased persistent sodium current (11).

Mutations in the voltage-gated potassium channel genes *KCNQ2* and *KCNQ3* are associated with benign familial neonatal convulsions (BFNC). This syndrome is characterized by clusters of seizures in the first days of life and remission within the first year (12-14). *KCNQ2* and *KCNQ3* encode Kv7.2 and Kv7.3 respectively, which heterodimerize to form a slowly activating and inactivating voltage-gated potassium channel. These channels generate M current, important for controlling repetitive firing upon strong excitatory stimulation (15, 16). The $Kcnq2^{V182M/+}$ line was generated by ethylnitrosourea (ENU) mutagenesis and carries the amino acid substitution in the third transmembrane segment of *Kcnq2*. Heterozygous $Kcnq2^{V182M/+}$ mutants exhibit reduced thresholds for minimal clonic seizures and rare spontaneous seizures as adults even though they have a normal lifespan (17). We previously showed genetic interaction between *Scn2a* and *Kcnq2* in mice (17).

SCN8A mutations are associated with ataxia and behavioral abnormalities in humans and movement disorders in mice. Mice homozygous for the *Scn8a*$^{med\text{-}jo}$ missense mutation exhibit tremor and cerebellar ataxia. Although *Scn8a*$^{med\text{-}jo}$ heterozygous mutants have no visible abnormalities, they do have spontaneous spike-wave discharges characteristic of non-convulsive absence seizures (18-21). We recently found that the loss-of-function mutations in heterozygous *Scn8a*$^{med\text{-}jo}$ mutants and heterozygous *Scn8a*$^{med/+}$ mutants were more resistant to flurothyl and kainic acid induced seizures. We also showed that the *Scn8a*$^{med\text{-}jo}$ allele could rescue the reduced seizure threshold and premature lethality of heterozygous *Scn1a* knockout (*Scn1a*$^{KO/+}$) mice, suggesting that *Scn8a* may play an important role in the excitatory circuits that influence convulsive seizure thresholds (22).

Unrelated individuals with GEFS+ exhibit a wide range of epilepsy subtypes and severities that may reflect, in part, the relative effect of different *SCN1A* mutations on channel function. Similar variability is also seen between affected family members who carry the same *SCN1A* mutation (23, 24). This suggests that in addition to the primary mutation, the clinical manifestation of epilepsy can be influenced by other factors such as stochastic events during development, environmental influences or genetic modifiers. Mouse models with sodium channel mutations exhibit variable phenotypes depending on the genetic background, supporting a role for genetic modifiers (17, 25-27). Based on these observations, we hypothesize that genetic modifier loci may contribute to the variable clinical presentation observed in GEFS+.

To test the hypothesis that genetic modifiers can contribute to GEFS+ variability, we examined the effect of mutations in *Scn2a*, *Kcnq2* and *Scn8a* on the epilepsy

phenotype of the *Scn1a*$^{R1648H/+}$ mouse model. Here we demonstrate that mutations in *Scn2a* and *Kcnq2* exacerbate the phenotype, whereas altered *Scn8a* function ameliorates it. Our results provide support for genetic modification as one mechanism by which the clinical presentation of GEFS+ can be altered, indicating that neuronal excitability is influenced by the net activity of ion channels.

Materials and Methods

Mice

Kcnq2$^{V182M/+}$ mice were generated by ENU mutagenesis (http://nmf.jax.org) at The Jackson Laboratory (Jackson Labs stock #004703). *Kcnq2*$^{V182M/+}$ heterozygous mutants are maintained by continued backcrossing to C57BL/6J (B6) background strain (Jackson Labs stock #000664). Q54 transgenic mice congenic on the B6 strain (B6.Q54) were established as described and are maintained by continued backcrossing of hemizygous B6.Q54 males to B6 females (Jackson Labs stock #000664) (25). *Scn8a*$^{med-jo}$ mice were obtained from The Jackson Laboratory and are maintained on the B6 background (Jackson Labs stock #003798). *Scn1a*$^{R1648H/+}$ mice were generated as previously described (7). *Scn1a*$^{R1648H/+}$ mice on a mixed 129S6.C57BL/6J(N2–3) background were used for mating with *Scn8a*$^{med-jo}$ mice. *Scn1a*$^{R1648H/+}$ mice on the 129S6/SvEvTac (129) background were used for mating with B6.Q54 and B6.*Kcnq2*$^{V182M/+}$ mice. 129.*Scn1a*$^{R1648H/+}$, B6.Q54, B6.*Kcnq2*$^{V182M/+}$ and *Scn8a*$^{med-jo}$ mice were genotyped as previously described (7, 11, 17, 22).

Generation of Double Mutant Mice

Double heterozygous mutants were generated by crossing 129.*Scn1a*$^{R1648H/+}$ females with B6.*Kcnq2*$^{V182M/+}$ or B6.Q54 males generating F1.*Scn1a*$^{R1648H/+}$;*Kcnq2*$^{V182M/+}$ or F1.*Scn1a*$^{R1648H/+}$;Q54 mice and F1 control littermates. *Scn1a*$^{R1648H/+}$ females on a mixed B6/129 background were used for mating to *Scn8a*$^{med\text{-}jo}$ males. *Scn1a*$^{R1648H/R1648H}$;*Scn8a*$^{med\text{-}jo}$ mutants and controls were generated by crossing *Scn1a*$^{R1648H/+}$;*Scn8a*$^{med\text{-}jo}$ males with *Scn1a*$^{R1648H/+}$ females. All double mutants were obtained at expected Mendelian ratios. Littermates were used for all experiments to minimize variation due to differences in genetic background. Mice were housed in pathogen-free mouse facilities with 12-h light/dark cycles. Food and water were available *ad libitum*. All experimental protocols were approved by the Emory University and Vanderbilt University IACUC committees.

Flurothyl Seizure Induction

Mice between eight and twelve weeks of age were placed in a clear Plexiglas chamber and flurothyl (2,2,2-trifluroethylether) was slowly introduced into the chamber via a syringe pump at a rate of 20 µl/min and allowed to volatilize (Sigma-Aldrich). Seizure thresholds were determined by measuring latency to the first myoclonic jerk (MJ) and generalized tonic-clonic seizure (GTCS). The MJ is the first observable behavioral response, characterized by a brief jerk of the shoulders and/or neck. The GTCS is characterized by convulsions of the entire body and loss of posture. Data from males and females were analyzed separately. No sex differences were observed therefore data from both sexes were combined. Statistical analysis between genotypes was performed using

one-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference (PLSD) *post hoc* test.

Video-ECoG Monitoring

Mice were implanted with prefabricated headmounts for video-Electrocorticography (ECoG) monitoring (Pinnacle Technology, Inc.). Briefly, mice were anesthetized with isoflurane and placed in a stereotaxic frame and headmounts were attached to the skull with four stainless steel screws that serve as cortical surface electrodes (Kopf). Headmounts were positioned 0–0.5 mm posterior to lambda. The anterior screw electrodes were 0.5–1 mm posterior to bregma and 1 mm lateral from the midline. The posterior screws were 4.5–5 mm posterior to bregma. After ≥ 24 hours of recovery, mice were placed in a Plexiglas bowl (14" h×16" diameter) and ECoG data were collected from freely moving mice. Acquisition and analysis of digitized data was performed with Sirenia software along with contemporaneous video recordings (Pinnacle Technology, Inc.). Epileptiform activity was scored manually.

Results

Q54 and Kcnq2$^{V182M/+}$ Alleles Exacerbate the Phenotype of Scn1a$^{R1648H/+}$ Mutants

To model the effect of inheriting mutations in sodium channel genes *Scn1a* and *Scn2a*, we generated F1.*Scn1a*$^{R1648H/+}$;Q54 double mutants by crossing 129.*Scn1a*$^{R1648H/+}$ females with B6.Q54 males. Sporadic death of F1.*Scn1a*$^{R1648H/+}$;Q54 double mutants began to occur at postnatal day 16 (P16) with 100% mortality by P24 (Figure 4.1).

Beginning at postnatal day 16 (P16), F1.*Scn1a*$^{R1648H/+}$;Q54 double mutants exhibited spontaneous focal motor seizures and GTCS. In 137 hours of ECoG recording, we observed 25 GTCS and 11 focal motor seizures (Table 4.1; Figure 4.2B). Generalized seizures lasted 45–100 s, during which time mice experienced repetitive jerking of all four limbs and neck, running, jumping and tail clonus. These seizures often ended with tonic hindlimb extension, indicative of a severe seizure. During ECoG recording we observed two F1.*Scn1a*$^{R1648H/+}$;Q54 mice that had severe seizures with hindlimb extension followed by death. Focal motor seizures lasted 10 s and were characterized by forelimb clonus. F1.Q54 littermates displayed a similar number of focal motor seizures. However, GTCS were rare in F1.Q54, with only one observed during 168 hours of ECoG recordings (Table 4.1). We saw no seizures in F1.*Scn1a*$^{R1648H/+}$ littermates (Table 4.1, Figure 4.2A). This was expected as previous monitoring of three to five month old *Scn1a*$^{R1648H/+}$ mice detected spontaneous seizures with a low average frequency of one seizure per 64 hours of recording (7).

To model the effect of inheriting mutations in *Scn1a* and *Kcnq2*, we generated F1.*Scn1a*$^{R1648H/+}$;*Kcnq2*$^{V182M/+}$ double heterozygous mutants by crossing 129.*Scn1a*$^{R1648H/+}$ females with B6.*Kcnq2*$^{V182M/+}$ males. At P19, sporadic death of F1.*Scn1a*$^{R1648H/+}$;*Kcnq2*$^{V182M/+}$ mice began to occur, with 42% mortality by P25, demonstrating the ability of the *Kcnq2*^{V182M} allele to exacerbate the phenotype of *Scn1a*$^{R1648H/+}$ mice (Figure 4.1). Interestingly, 47% of F1.*Scn1a*$^{R1648H/+}$;*Kcnq2*$^{V182M/+}$ double heterozygous mutants survived for more than 100 days (Figure 4.1).

At P16, F1.*Scn1a*$^{R1648H/+}$;*Kcnq2*$^{V182M/+}$ mice began to display spontaneous generalized seizures. In 330 hours of ECoG recording, we observed 87 MJ, four GTCS

and one focal motor seizure in F1.*Scn1a*$^{R1648H/+}$;*Kcnq2*$^{V182M/+}$ double heterozygous mutants (Table 4.1; Figure 4.2C). During a generalized seizure the mice typically experienced repetitive jerking of all four limbs and neck, running, jumping and tail clonus. Generalized seizures were periodically followed by tonic extension of the hindlimbs. During ECoG recording we observed two F1.*Scn1a*$^{R1648H/+}$;*Kcnq2*$^{V182M/+}$ mice that had severe seizures with hindlimb extension followed by death. Epileptiform events were not observed in F1.*Scn1a*$^{R1648H/+}$ or F1.*Kcnq2*$^{V182M/+}$ control littermates (Table 4.1).

Table 4.1 Recorded ECoG Events from F1 Double Mutant Mice

Video ECoG data was collected from freely moving mice. Double mutants and single mutant control littermates (n ≥ 3) were observed in 12 hour intervals. Data acquisition and analysis were performed with contemporaneous video. Epileptiform activity was manually scored.

Genotype	Events			*n*	Age Range (Days)	Total Hours of ECoG monitoring
	GTCS	Partial	MJ			
F1.*Scn1a*$^{R1648H/+}$;Q54	25	11	3	5	17-21	137
F1.*Scn1a*$^{R1648H/+}$; *Kcnq2*$^{V182M/+}$	4	1	87	7	18-36	330
F1.*Scn1a*$^{R1648H/+}$	0	0	0	4	18-42	240
F1.Q54	1	14	0	3	17-25	168
F1.*Kcnq2*$^{V182M/+}$	0	0	0	3	22-44	350

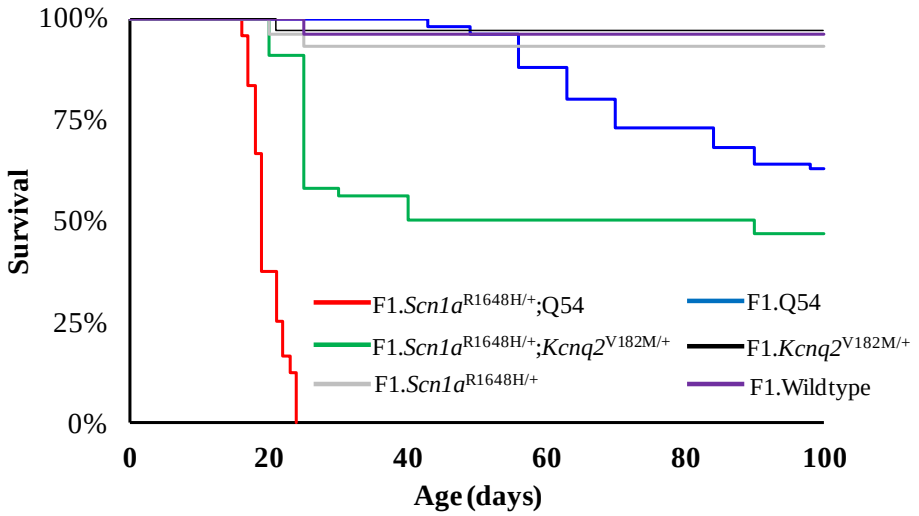

Figure 4.1 Survival of F1 Double Mutant and Control Littermates

Survival of F1 double mutant mice and littermate controls are shown. Survival was monitored until 100 days of age. All genotypes had n ≥ 24.

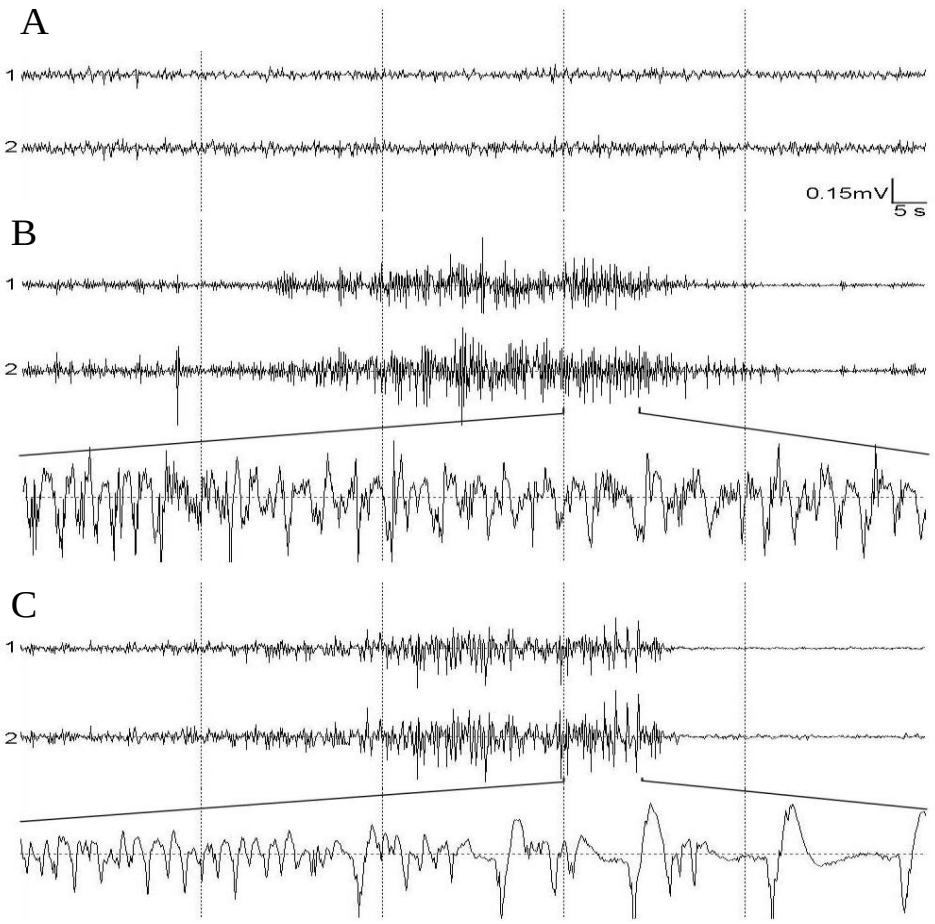

Figure 4.2 Q54 and *Kcnq2*$^{V182M/+}$ Alleles Exacerbate the *Scn1a*$^{R1648H/+}$ Phenotype
A. Representative normal ECoG pattern from a control F1.*Scn1a*$^{R1648H/+}$ heterozygote mouse. B. Representative ECoG recording from a F1.*Scn1a*$^{R1648H/+}$;Q54 double heterozygous mutant during a seizure. C. Representative ECoG recording from a F1.*Scn1a*$^{R1648H/+}$;*Kcnq2*$^{V182M/+}$ double heterozygous mutant during a seizure. Channel 1 recorded from right posterior to left posterior. Channel 2 recorded from right anterior to left posterior.

Scn8a Dysfunction Restores Normal Seizure Thresholds in Scn1a$^{R1648H/+}$ *Mutants*

We previously demonstrated that two heterozygous *Scn8a* mutants, *Scn8a*$^{med\text{-}jo}$ and *Scn8a*^{med}, exhibited increased resistance to flurothyl and kainic acid induced seizures (22). In contrast, *Scn1a*$^{R1648H/+}$ mutants had reduced thresholds to flurothyl induced GTCS (7). To investigate whether the *Scn8a*$^{med\text{-}jo}$ allele could alter convulsive seizure thresholds in *Scn1a*$^{R1648H/+}$ mice, we generated double heterozygous *Scn1a*$^{R1648H/+}$;*Scn8a*$^{med\text{-}jo}$ mutants. Thresholds to flurothyl induced seizures were

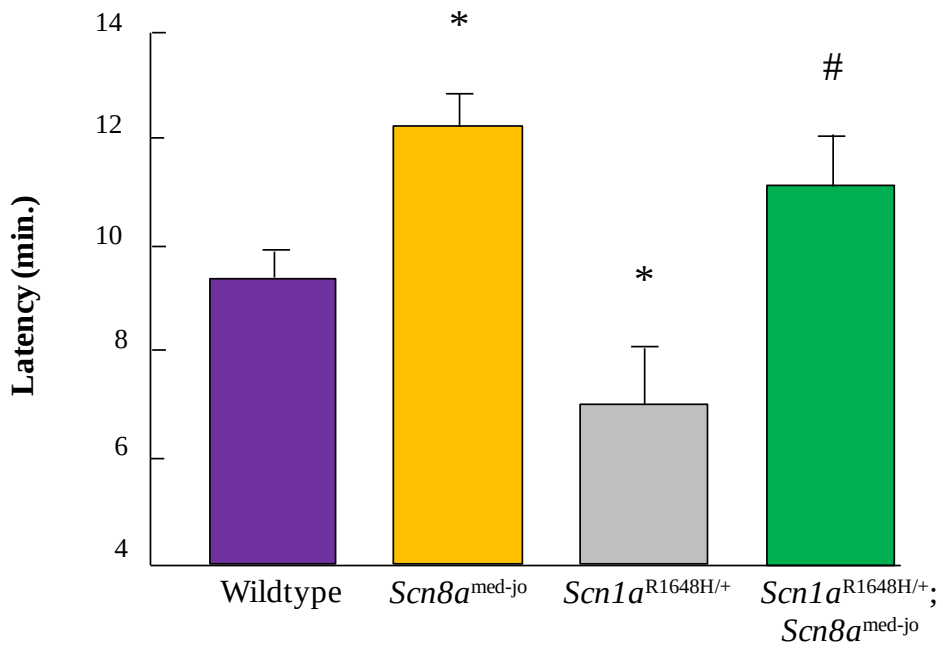

Figure 4.3 Latency to Flurothyl Induced Seizures
Average latency in minutes to the first GTCS is shown. Group sizes were n=9-10. Significant differences between groups were determined by ANOVA: $F_{(3,35)}= 9.858$, $p < 0.001$. P-values were determined by Fisher's PLSD *post hoc* test. * indicated $p < 0.05$ compared to wildtype littermates. # indicates $p < 0.001$ compared with *Scn1a*$^{R1648H/+}$ mutants. Error bars represent SEM.

compared between *Scn1a*$^{R1648H/+}$;*Scn8a*$^{med\text{-}jo}$ double mutants and control single heterozygote and wildtype littermates, which revealed a significant effect by ANOVA ($F_{(3,35)}= 9.858$, $p < 0.001$) . In agreement with our previous observations, the average latency to flurothyl-induced GTCS in *Scn8a*$^{med\text{-}jo}$ mutants was 30% longer compared to wildtype littermates (Figure 4.3) (7, 22). *Post hoc* analysis of the increased latency to GTCS experienced by *Scn8a*$^{med\text{-}jo}$ compared to wildtype littermates was statistically significant ($p < 0.05$). In contrast, a 21% reduction was observed in *Scn1a*$^{R1648H/+}$ mutants when compared to wildtype littermates, which was statistically significant ($p < 0.05$) by *post hoc* analysis (Figure 4.3). The average latency to GTCS of *Scn1a*$^{R1648H/+}$;*Scn8a*$^{med\text{-}jo}$ double mutants was 50% longer compared to *Scn1a*$^{R1648H/+}$ mutants. *Post hoc* analysis revealed the increased latency to GTCS observed in *Scn1a*$^{R1648H/+}$;*Scn8a*$^{med\text{-}jo}$ double

mutants was statistically significant when compared to *Scn1a*$^{R1648H/+}$ mutants ($p < 0.001$), but not statistically different from wildtype littermates ($p > 0.05$). These results demonstrate seizure thresholds can be restored to more normal levels in *Scn1a*$^{R1648H/+}$ mice by altering the function of *Scn8a*.

Scn8a$^{med\text{-}jo}$ *Mutation Prolongs the Lifespan of Scn1a*$^{R1648H/R1648H}$ *Mutants*

To determine whether the presence of the *Scn8a*$^{med\text{-}jo}$ allele could improve the survival of homozygous *Scn1a*$^{R1648H/R1648H}$ mice, we compared the lifespans of *Scn1a*$^{R1648H/R1648H}$ and *Scn1a*$^{R1648H/R1648H}$;*Scn8a*$^{med\text{-}jo}$ littermates. Similar to previous observations, *Scn1a*$^{R1648H/R1648H}$ mutants exhibited 50% mortality by P19.5 and 100% lethality by P25 (Figure 4.4) (7). In contrast, only 25% mortality was observed for *Scn1a*$^{R1648H/R1648H}$;*Scn8a*$^{med\text{-}jo}$ mice at P25 and 47% of these mutants survived for over 100 days, a significant ($p < 0.001$) improvement compared to controls (Figure 4.4).

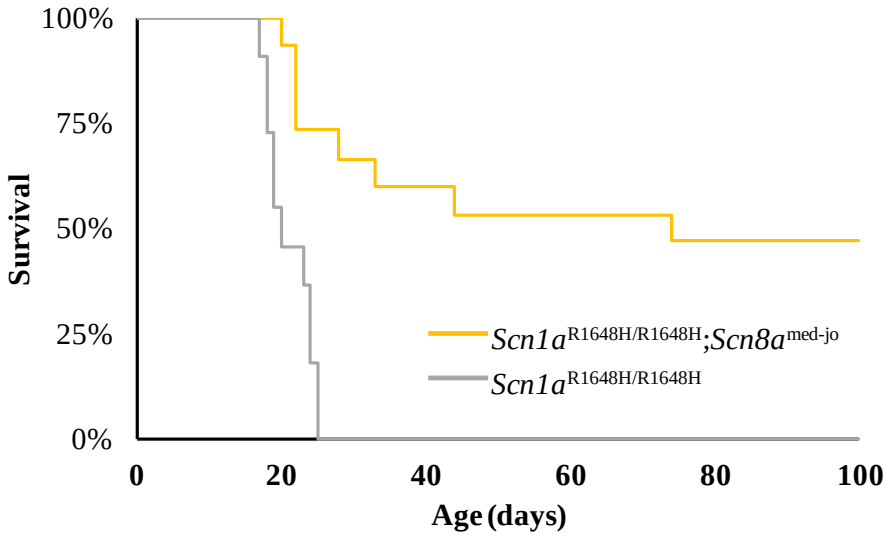

Figure 4.4 Survival of *Scn1a*$^{R1648H/R1648H}$;*Scn8a*$^{med\text{-}jo}$ and Control Littermates
Survival of double mutant *Scn1a*$^{R1648H/R1648H}$; *Scn8a*$^{med\text{-}jo}$ mice and littermate *Scn1a*$^{R1648H/R1648H}$ controls are shown. Survival was monitored until 100 days of age. All genotypes had n = 11-16.

Discussion

One characteristic feature of GEFS+ is the wide range of seizure types and severities frequently seen among family members with the same *SCN1A* mutation (23, 24, 28). Based on these observations, we hypothesized that the variable clinical presentation in GEFS+ is possibly due to contributions from additional genetic modifiers.

Even though it is well recognized that genetic modifiers can influence the clinical presentation of a disorder, we know of relatively few human modifier genes. Cystic fibrosis (CF) represents one good example of a monogenic human disease with a known genetic modifier. CF results from recessive mutations in CFTR, a cAMP-dependent chloride channel (29). Multiple studies have shown a significant association between two transforming growth factor-β (TGF-β) polymorphic variants and lung disease severity in CF (30, 31). TGF-β is an inflammatory cytokine and directly inhibits CFTR function (32). As a result, polymorphisms that increase circulating TGF-β levels are more common in patients with severe lung disease.

Although genetic interactions have been difficult to demonstrate in epilepsy patients, model organisms can help in the search for genetic modifiers of seizure severity. We previously demonstrated that mutations in other ion channels could modify spontaneous seizure activity and lifespans of *Scn1a*$^{KO/+}$ and Q54 mutants (17, 22). In the presence of the *Scn8a*$^{med\text{-}jo}$ allele, the severe seizure phenotype of *Scn1a*$^{KO/+}$ mice was dramatically ameliorated (22). In contrast, the *Kcnq2*$^{V182M/+}$ and *Szt1* mutations exacerbated the epilepsy phenotype of Q54 mice (17).

Here we demonstrated *Scn2a*, *Kcnq2* and *Scn8a* mutant alleles modified the phenotype of *Scn1a*$^{R1648H/+}$ mice. This supports genetic modification as one mechanism

by which the clinical presentation of GEFS+ can be altered. The genetic interactions between these ion channels imply that variants within *Scn2a, Kcnq2* and possibly *Kcnq3,* may exacerbate the clinical presentation of GEFS+, shifting it to a more severe part of the GEFS+ spectrum. As previously observed with *Scn1a*$^{KO/+}$ mutants, the *Scn8a*$^{med\text{-}jo}$ allele could rescue the increased susceptibility to flurothyl induced GTCS in *Scn1a*$^{R1648H/+}$ mice by raising seizure thresholds to a level comparable to wildtype littermates (22). In addition, while *Scn1a*$^{R1648H/R1648H}$ mutants do not survive more than 26 days, 47% of *Scn1a*$^{R1648H/R1648H}$; *Scn8a*$^{med\text{-}jo}$ mutants were still alive after 100 days. These results demonstrate that altered *Scn8a* function is capable of compensating for abnormalities in neuronal excitability caused by *Scn1a* mutations. These results suggest that selective blocking of *Scn8a* may be similarly protective in patients with epilepsy. Interestingly, although *Scn8a*$^{med\text{-}jo}$ heterozygotes have increased resistance to convulsive seizures, we recently reported they do have spontaneous absence seizures, characterized by hypersynchrony of the thalamocortical system (20). This underscores the idea that the net effect of sodium channel dysfunction in different neuronal circuits is highly dependent on the channel composition and synaptic function within each circuit.

In contrast, whereas *Scn1a*$^{R1648H/+}$ mice have infrequent, adult-onset generalized seizures, the presence of either the Q54 or *Kcnq2*$^{V182M/+}$ alleles resulted in severe, juvenile-onset generalized seizures and shortened lifespan. Recordings of neurons isolated from *Scn1a* mutant mice suggest there is decreased GABAergic neurotransmission in the hippocampus and cortex (7, 26, 27). In F1. *Scn1a*$^{R1648H/+}$;Q54 double mutants, the combination of increased excitability of pyramidal neurons due to the *Scn2a* mutation and decreased inhibition from the *Scn1a* mutation resulted in severe

generalized seizures and lethality. Reduced GABAergic inhibition may prevent localized seizure termination, resulting in secondary generalization of seizures and the more severe phenotype. Similarly, it was demonstrated that loss of M-current in *Kcnq2* mutants resulted in hyperexcitability of hippocampal pyramidal CA1 neurons, which in combination with reduced GABAergic inhibition, may permit secondary generalization of seizures in double F1. *Scn1a*$^{R1648H/+}$; *Kcnq2*$^{V182M/+}$ mutants (33, 34). Alternatively, because these genes have widespread expression in the brain, it is possible that the severe phenotype observed in double mutant mice was the result of primary generalized seizures.

Given that the *Scn1a*$^{R1648H/R1648H}$;*Scn8a*$^{med\text{-}jo}$ and F1.*Scn1a*$^{R1648H/+}$;*Kcnq2*$^{V182M/+}$ compound mutants were on mixed genetic backgrounds, the respective 47% and 42% survival rates observed raises the possibility that genetic variants which differ between the B6 and 129 inbred strains may influence disease severity. Alternatively, the increased mortality between P20 and P40 in the double mutant heterozygotes may reflect a more vulnerable juvenile stage due to ongoing maturation processes in the developing brain. In humans, *KCNQ2* dysfunction leads to BFNC, an epilepsy disorder affecting neonates that typically remits by one year of age. The spontaneous remission of BFNC appears to correlate with brain maturation (15). However, it is also plausible that the observed survival rates are due to stochastic events.

Together with our previous study analyzing the B6.Q54;*Kcnq2*$^{V182M/+}$ mice, our observations from F1. *Scn1a*$^{R1648H/+}$;*Kcnq2*$^{V182M/+}$ mutants illustrate that M-channel dysfunction in a background of abnormal excitability promotes seizure initiation and increases seizure severity. This suggests that increasing M-current may be of therapeutic

benefit in individuals with generalized epilepsy. The M-current enhancer ezogabine has therapeutic benefit as an adjunctive therapy in patients with drug-resistant focal epilepsy (35-37)

Our findings show that voltage-gated ion channel variants can modify the phenotype of a mouse model of GEFS+ and therefore suggest that coding and possibly noncoding variants in *Scn2a, Scn8a* and *Kcnq2* may influence clinical presentation and severity in patients with *SCN1A* mutations. The demonstrated genetic interactions between *Scn1a*, *Scn2a*, *Scn8a* and *Kcnq2*, together with previous reports showing a genetic interaction between *Scn2a* and *Kcnq2*, and *Kcna1* and *Cacna1a,* support the notion that neuronal firing patterns are determined by the net sum of voltage-gated ion channel activity (17, 38). Hence, screening patients for mutations in a panel of selected ion channel genes may improve the utility of molecular diagnosis for risk assessment and guiding disease treatment. Traditional single gene screening approaches will be replaced by next-generation sequencing technologies, which will enable targeted re-sequencing of panels of selected genes, or whole exome analysis. Clinical diagnostic testing using next-generation sequencing of gene panels is already being applied to epilepsy genetics (GeneDx) and are likely to replace single gene testing in the near future.

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CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

Summary

Epilepsy is a common neurological disease, affecting almost 3 million Americans and 1% of the worldwide population (1). Considered a spectrum disorder, epilepsy has more than 25 associated syndromes. These range from the severe and debilitating Dravet and Lennox Gastaut syndromes to the mild and treatable conditions of GEFS+ (Genetic Epilepsy with Febrile Seizure Plus) and absence epilepsy (2-4).

Two-thirds of patients diagnosed with epilepsy have no known cause for their disease, however recent evidence suggests most result from complex genetic interactions (5, 6). Mutations identified in nicotinic acetylcholine and GABA receptors, chloride channels and voltage-gated potassium and sodium channels are known to be responsible for monogenic epilepsies (7). Identification of these genes provided clues about the etiology of common epilepsy syndromes with more complex inheritance.

Mutations in voltage-gated sodium channels are responsible for several types of human epilepsy, including GEFS+, Benign Familial Neonatal-Infantile Seizures (BFNIS), Dravet and Ohtahara syndromes (8-10). Family members who inherit the same epilepsy-associated sodium channel mutation frequently have clinical phenotypes that vary dramatically. This is seen in both GEFS+ and Dravet syndrome. This suggests that

other factors, possibly genetic, modify the primary mutation, resulting in a more or less severe phenotype.

Mouse models have been generated to study the genetic basis of epilepsy. Often, the genetic strain background alters the disease phenotype, suggestive of genetic modifiers in epilepsy. The *Scn2a*^{Q54} (Q54) transgenic mouse model has a gain-of-function mutation that results in persistent sodium current and epilepsy (11). On the C57BL/6J (B6) strain background, B6.Q54 mice have infrequent, adult-onset focal motor seizures and a 75% survival rate beyond six months of age (12). When crossed with the SJL/J (SJL) strain, the resulting F1.Q54 offspring experience high seizure frequency with juvenile onset and less than 25% survival until six months of age (12). We mapped two dominant loci that modify seizure susceptibility in Q54, designated *Moe1* (Modifier of Epilepsy) on chromosome 11 and *Moe2*, on chromosome 19 (12).

Chapter II focused on fine-mapping and narrowing of the *Moe1* interval. We utilized interval-specific congenic lines spanning the *Moe1* locus and RNA-Seq to identify candidate modifier genes of the Q54 seizure phenotype. *Cacna1g* and *Hlf* were selected among the top *Moe1* candidates.

Cacna1g, encoding the T-type calcium channel α_1 subunit, was prioritized as a top female candidate modifier gene of the *Moe1* locus based on previous research attributing *Cacna1g* mutations to human and mouse epilepsy (13-15). *Cacna1g* was hypothesized to modify the Q54 seizure phenotype based on the significant strain-specific differences in expression profiles of transcripts which have functional kinetic diversity (13). *Cacna1g* channels preferentially expressed in SJL females reduce

synchronous thalamocortical oscillations, a common feature of epilepsy, while B6 females express *Cacna1g* channels prone to hyperexcitability (13, 16-18).

Hlf, encoding hepatic leukemia factor, a PAR bZIP transcription factor, was prioritized as a top candidate modifier gene of the *Moe1* locus in males. This was based on previous research of a PAR bZIP transcription factor family knockout mouse model that experienced spontaneous GTCS and absence seizures (19). Further analysis of the model revealed a reduction in PDXK, a key enzyme involved in the conversion of pyridoxine to pyridoxal 5' phosphate (PLP) (19). PLP is a key coenzyme involved in amino acid and neurotransmitter metabolism, including GABA and glutamate (20). PLP has a substantial connection with human and mouse epilepsy (20-22). *Hlf* was hypothesized to modify the Q54 seizure phenotype by contributing to strain-specific PDXK and PLP levels (13). *Hlf* is expressed at a significantly reduced level in B6 males compared to SJL (13). Furthermore, we observed a trend toward significant reduction ($p=0.135$) of PDXK expression in B6 males compared to SJL by RNA-Seq (N. Hawkins and J. Kearney, unpublished observations). These reductions in B6 may decrease PLP levels, consequently increasing seizure susceptibility.

Chapter III focused on assessing the modifier potential of *Cacna1g* and *Hlf.* Through generation of *Cacna1g* BAC transgenic (SJL.1G5) and *Hlf* targeted knockout (B6.*Hlf* $^{KO/+}$) animal models, we systematically evaluated the ability of *Cacna1g* transgenic expression or loss of *Hlf* to alter seizure severity of the Q54 phenotype.

We tested the candidate modifier gene *Cacna1g* by crossing the BAC transgenic SJL.1G5 line with B6.Q54 to generate double mutant mice. Double transgenic F1.1G5;Q54 males, not females, had significantly more seizures than F1.Q54 controls.

Although data from Chapter II suggested *Cacna1g* was a female-specific modifier, results from female F1.1G5;Q54 mice were difficult to interpret due to the possibility that the transgene may be subject to X-inactivation (13). These results suggest *Cacna1g* is a genetic modifier of the male F1.Q54 phenotype. Future evaluation of additional BAC transgenic lines with autosomal integration of the transgene will confirm if *Cacna1g* is a genetic modifier of the female F1.Q54 phenotype.

To evaluate the contribution of *Hlf* on the seizure phenotype of Q54 mice, we generated double mutant mice on two genetic strain backgrounds by crossing B6.*Hlf* $^{KO/+}$;Q54 males with B6.*Hlf* $^{KO/+}$ or SJL females. On the F1 strain, no significant differences in seizure frequency or survival were observed between heterozygous knockout F1.*Hlf* $^{KO/+}$;Q54 mice and F1.Q54 controls in either sex. On the B6 strain, double mutant homozygous knockout B6.*Hlf* $^{KO/KO}$;Q54 males experienced GTCS not traditionally seen in the B6.Q54 model. Although no overt seizure phenotype was observed in either heterozygous B6.*Hlf* $^{KO/+}$;Q54 or homozygous B6.*Hlf* $^{KO/KO}$;Q54 females, these mice were born at significantly reduced ratios than expected. The ratio shift suggests reduced viability of B6.*Hlf* $^{KO/KO}$;Q54 females. Overall, these results suggest that total loss of *Hlf* is detrimental to the B6.Q54 phenotype.

In addition to seizure analysis, we evaluated our *Hlf* hypothesis by testing the effects of altering dietary pyridoxine on the Q54 phenotype. We hypothesized that a pyridoxine deficient diet would increase seizure frequency, based on past data which demonstrated that mice maintained on a deficient diet experienced increased seizure frequency (23-25). B6.Q54 females on the pyridoxine deficient diet had increased seizure frequency and reduced survival compared to controls. While the B6.Q54 male seizure

phenotype was not altered, neurochemistry studies showed B6.Q54 males maintained on the deficient diet had significant levels of proline detected in their brains, indicating an altered metabolic state. F1.Q54 males maintained on the deficient diet trended toward increased seizure frequency, while there was no change in F1.Q54 females. Neurochemistry studies showed F1.Q54 males had significant changes in glutamate and GABA concentrations compared to controls. These results suggest that maintaining adequate dietary pyridoxine levels is important when there is an underlying epilepsy risk.

We hypothesized that a pyridoxine enriched diet would decrease seizure frequency, given that pyridoxine dependent epilepsies are successfully treated with PLP (21). No reduction in seizure frequency was observed in B6.Q54 males or females maintained on the pyridoxine enriched diet. F1.Q54 females maintained on the pyridoxine enriched diet had no changes in seizure frequency or survival. Paradoxically, F1.Q54 males had increased seizure frequency and reduced survival on the enriched diet compared to controls. These results suggest that dietary supplementation with pyridoxine megadoses offer no additional benefit.

Finally, *Hlf* was shown to modify the Dravet phenotype of *Scn1a*$^{KO/+}$ heterozygous knockout mice. By four weeks of age, only 45% of double heterozygous knockout F1.*Hlf* $^{KO/+}$;*Scn1a*$^{KO/+}$ mice survived compared to 71% of F1.*Scn1a*$^{KO/+}$ controls. This further supports *Hlf* as a genetic modifier of epilepsy as loss of *Hlf* alters two diverse epilepsy syndromes.

Chapter IV focused on a functional candidate gene approach to evaluate known epilepsy mutations Q54 and *Kcnq2*$^{V182M/+}$ on the phenotype of the *Scn1a*$^{R1648H/+}$ epilepsy model. To assess the modifier potential of Q54 and *Kcnq2*$^{V182M/+}$ mutations, we generated

double mutant mice and monitored seizure activity by ECoG and survival. Double mutant F1.*Scn1a*$^{R1648H/+}$;Q54 and F1.*Scn1a*$^{R1648H/+}$;*Kcnq2*$^{V182M/+}$ mice exhibited GTCS with 100% and 42% mortality by P25, respectively, unlike their single mutant control littermates. This suggests that genetic interaction between ion channels is one mechanism which contributes to the variable clinical presentation of inherited epilepsies.

Implications for Epilepsy

Complex genetics are likely responsible for the majority of epilepsy cases with no known origin (5, 6). Several Dravet and GEFS+ voltage-gated sodium channel gene mutations result in variable expressivity of an epilepsy phenotype. While some individuals have a severe, debilitating seizure diagnosis, many have remitting seizures or no phenotype at all. This trend is likely a result of complex inheritance of protective and risk alleles in multiple genes. Mildly affected patients have possibly inherited protective alleles that reduced or prevented seizure activity. Alternatively, individuals with a more devastating phenotype likely inherited risk alleles that altered network function and resulted in a hyperexcitable, seizure-prone brain. Hypothesized epilepsy modifier genes responsible for the altered phenotype are relatively unknown. Our lab previously identified a modifier gene in mice that was subsequently shown to contribute to epilepsy risk in human patients (26). *Kcnv2*, encoding the voltage-gated potassium subunit Kv8.2, was identified as a modifier gene that influences the Q54 epilepsy phenotype (26). *Kcnv2* forms functional potassium channels by complexing with another potassium channel subunit, *Kcnb1*, which encodes Kv2.1, the major contributor to delayed rectifier potassium current (26). This Kv8.2/Kv2.1 heteromeric configuration results in altered

delayed rectifier channel kinetics (27, 28). In human epilepsy patients, two *KCNV2* missense variants were detected and when coexpressed with *KCNB1*, resulted in increased suppression of delayed rectifier potassium currents (26). Recently, a *KCNB1* variant was identified by exome sequencing of infantile spasm and Lennox-Gastaut families (29). *Hlf* was identified as a candidate modifier of the Q54 epilepsy phenotype due to its hypothesized influence on the pyridoxine pathway (13, 19). Traditional pyridoxine dependent epilepsies are defined by severe neonatal seizures that are successfully treated with pyridoxine or PLP, not classical AEDs (30). Recent research revealed a "hidden" pyridoxine deficient epilepsy patient whose phenotype resembled Dravet syndrome, which is uncharacteristic of pyridoxine dependent epilepsies (30). The patient was screened for two genes in the pyridoxine pathway, *ALDH7A1* and *PNPO*. (30). A pathogenic mutation was identified in *ALDH7A1*, suggesting some unexplained epilepsies may result from pyridoxine deficiencies (30). This further supports *Hlf* as a candidate modifier gene of an epilepsy phenotype as mutations in *Hlf* may reduce the efficiency of the pyridoxine pathway and thus increase seizure susceptibility. This demonstrates that identification of mouse epilepsy modifier genes can directly influence discovery of human epilepsy modifier genes.

We have highlighted several examples throughout Chapters I-IV demonstrating that exacerbated or ameliorated epilepsy phenotype result from combining mutations from two different genes. This suggests that variants in multiple genes may modify the clinical phenotype in human epilepsy. This is particularly important for advancements in diagnostic and long-term prognostic capabilities as well as in designing novel therapeutic targets. Patients can wait months or even years to receive a specific epilepsy diagnosis.

While genetic testing in epilepsy is more accessible, especially in the more severe epileptic encephalopathies such as Dravet syndrome, its full impact has not yet been attained. Recent exome sequencing projects have started to identify novel genes responsible for epilepsy with a previously unknown origin, but exome sequencing is not yet routinely used in the clinic (9, 29, 31). Moreover, even if a hypothesized disease-causing mutation in a gene is detected, prognosis is uncertain. For example, mutations in *SCN1A* can lead to the devastating Dravet syndrome or the very treatable GEFS+. Complicating things further is the phenotypic variability often seen in patients with the same mutation.

By identifying genetic modifiers of epilepsy in mice, including *Cacna1g* and *Hlf,* improvements in both patient diagnosis and prognosis can occur. While genetic tests often include calcium channels, screening for variants in calcium and additional ion channels in the same patient may predict a possible severe seizure phenotype. Furthermore, adding modifier genes to genetic screens, including *Hlf,* may advance prognosis efficiency. Detection of a primary mutation with additional variants in modifier genes may suggest a disease course. For example, if a *SCN1A* positive GEFS+ patient also has an *Hlf* variant, we might predict a more severe phenotype. Alternatively, if the patient has inherited a protective modifier allele, we could predict a mild phenotype.

Identification of epilepsy modifier genes can also advance therapeutics. Current antiepileptic medications mainly target GABA receptors, voltage-gated sodium channels and calcium channels (32). A third of epilepsy patients are unable to attain seizure control using these medications (33). For patients who are seizure free, many have tried at least two drugs before achieving seizure control (33, 34). Furthermore, controlled epilepsies

frequently are a result of combination therapy, with patients taking at least two drugs simultaneously (33, 34). The pharmacoresistance observed in epilepsy patients is hypothesized to result from both genetic and environmental factors (35, 36). If we focus drug discovery efforts to non-traditional targets, like modifier genes, the novel therapeutics generated may alleviate seizure symptoms with reduced side effects. Since currently available drugs act on broad pathways like GABA, many patients experience extreme drowsiness or fatigue. Through targeting modifier genes, novel drugs may prevent seizures intrinsically rather than universally targeting and sedating brain activity.

To test *Cacna1g* as a potential drug target for epilepsy treatment, a high throughput screen to identify novel pharmacological inhibitors of *Cacna1g* would first be performed. Positive candidates from the screen would be further evaluated using whole-cell electrophysiology. Using HEK cells stably expressing *Cacna1g* , drug candidates would be assayed to identify those which inhibit calcium currents in a dose-dependent manner. The ideal drug would specifically target *Cacna1g*, therefore control experiments testing T, L, P, Q and N calcium channels is needed. Once a *Cacna1g* targeted drug is identified and validated *in vitro,* it can advance to *in vivo* testing. Essential experiments include seizure threshold testing to determine whether the drug raises the threshold to seizure induction compared to placebo treatment and EEG recordings of calcium channel mutants with epilepsy to determine if drug treatment reduces or prevents seizures.

To test *Hlf* as a potential drug target for epilepsy treatment, the molecular basis of *Hlf* function would first be determined. Currently, we hypothesize *Hlf* acts on the pyridoxine pathway, but it is unknown what this transcription factor regulates or where in the pathway it has its influence. Through the use of chromatin immunoprecipitation and

next-generation sequencing of immunoprecipated DNA (ChIP-seq), we can identify regions of the genome that are potentially targeted and bound by *Hlf*. Once these regions are determined, we can test the identified gene candidates to assess their influence on seizure susceptibility. We can generate a transgenic or knockout mouse model of each candidate gene to determine if spontaneous seizure activity occurs or if a reduced threshold to induced seizures exists. To further validate each model and bridge the candidate gene(s) and *Hlf* to the pyridoxine pathway, we can test if administration of PLP can prevent seizure activity or extend the threshold to induced seizures.

Verification of *Cacna1g* and *Hlf* as epilepsy modifier genes will suggest new targets for improved treatment of epilepsy and advance molecular diagnostic capabilities by identifying those patients who are most at-risk for developing severe epilepsy.

Future Directions

Investigation of additional candidate genes

We selected *Cacna1g* and *Hlf* as top priority candidate genes for evaluation of their potential modifier effects on the Q54 seizure phenotype. However other genes, including *Cacnb1*, *Slc25a39* and *Pnpo*, were also considered strong candidate modifier genes at the *Moe1* locus (Table 2.4). *Cacnb1* encodes a calcium β subunit and has previously been linked to epilepsy (37-39). RNA-Seq identified a significant expression difference in the *Cacnb1*-006 transcript between B6 and SJL females. Interestingly, the *Cacnb1*-006 transcript is thought to act as a long non-coding RNA (lncRNA) (13). To

evaluate the modifier potential of *Cacnb1*, a targeted *Cacnb1*-006 siRNA could be utilized to knockdown the lncRNA in the Q54 mouse model. Furthermore, we could determine the *Cacnb1* lncRNA targets to understand how it may affect excitability.

We also hypothesized a protective modifier gene is located within the *Moe1* locus (13). *Cltc* and *Dynll2* were suggested as top candidate genes within the protective region. By generating animal models overexpressing *Cltc* or *Dynll2*, we can evaluate if each gene has a protective effect on the Q54 phenotype. Furthermore, identification of protective alleles may provide novel therapeutic targets which have the ability to treat seizures with fewer side effects.

Comprehensive analysis of Cacna1g and Hlf modifier effects

To verify the full modifier potential of *Cacna1g*, it is necessary to generate and evaluate additional *Cacna1g* BAC transgenic lines. Recent pronuclear injections of the BAC clone containing *Cacna1g* (RP23-65I14) into B6 fertilized oocytes at the University of Michigan Transgenic Animal Model Core resulted in two transgenic founders, 1G3 and 1G6. BAC integrity was preliminarily analyzed, suggesting both transgenics have complete integration of the construct. At this time, the 1G3 male transgenic founder has transmitted the transgene to N1 offspring while the 1G6 female founder has not.

Hlf was verified as a modifier of the Q54 phenotype. Further investigation is needed to assess the mechanism of the *Hlf* modifier effect. Most importantly, ECoG analysis of homozygous B6.*Hlf* $^{KO/KO}$;Q54 and heterozygous B6.*Hlf* $^{KO/+}$;Q54 mice is crucial. This will confirm GTCS events seen during phenotyping as well as identify additional abnormal brain activity, like absence seizures. Female B6.*Hlf* $^{KO/+}$,

B6.*Hlf* $^{KO/+}$;Q54 and B6.*Hlf* $^{KO/KO}$;Q54 mice were born at significantly altered ratios than expected, suggesting reduced female viability. Future studies should include determining when B6.*Hlf* $^{KO/+}$;Q54 and B6.*Hlf* $^{KO/KO}$;Q54 females are being lost. *Hlf* was also identified as a modifier of survival of Dravet mice, an additional epilepsy model. To evaluate if F1.*Hlf* $^{KO/+}$;*Scn1a*$^{KO/+}$ mice are dying significantly earlier compared to controls due to increased seizure frequency or severity, ECoG recordings are necessary. Furthermore, we determined the B6.*Hlf* $^{KO/+}$ mouse model contains roughly ~45 Mb of 129 S2/SvPas ES cell DNA on chromosome 11. This is problematic, as it adds a third genetic strain into our mouse crosses. Due to the original 129 construct DNA being present, B6.*Hlf* $^{KO/+}$;Q54 and B6.*Hlf* $^{KO/KO}$;Q54 offspring are actually F1.(129 X B6) and homozygous 129, respectively, in *Moe1* instead of congenic B6. Moreover, from ISC line production (Chapter II) we are aware that multiple recombination "hot-spots" exist within *Moe1*. This may explain the inconsistency observed in seizure frequency and survival of B6.*Hlf* $^{KO/+}$;Q54 and B6.*Hlf* $^{KO/KO}$;Q54 mice. Offspring may have different regions of 129 present in *Moe1* due to recombination. To identify if recombination of B6 and 129 regions along chromosome 11 is responsible for the discrepancies observed in the *Hlf* $^{KO/+}$ X Q54 phenotyping crosses, genotyping must be performed on all phenotyped offspring. This will determine if recombination occurred and if so, we can categorize offspring into groups based on the amount of 129 DNA present.

Finally, to determine if these modifier genes are relevant to human epilepsy risk, it is essential to screen epilepsy patients for genetic variation in *HLF* and *CACNA1G*.This approach was utilized in our lab previously and validated the approach that identifying genetic modifier genes in mouse is translatable to human epilepsy gene detection (26).

Ohtahara syndrome as a result of Scn2a and Kcnq2 mutations

Since the publication of Hawkins *et al,* 2011 both *SCN2A* and *KCNQ2* have been implicated by whole genome sequencing in Ohtahara syndrome (9, 10, 40, 41). Hundreds of hours of ECoG data from F1.*Scn1a*$^{R1648H/+}$;Q54 and F1.*Scn1a*$^{R1648H/+}$;*Kcnq2*$^{V182M/+}$ offspring and controls are available for analysis. These offspring may serve as compelling mouse models of Ohtahara syndrome. For Hawkins *et al,* the focus was to identify GTCS, absence and focal seizures. We did not examine ECoG data for other irregular features such as classic Ohtahara suppression burst patterns. Furthermore, brain histology was not performed on offspring. Ohtahara patients usually have severe brain abnormalities, including atrophy of the cortex, corpus callosum and cerebellum (10, 10, 42). Future studies should inspect ECoG data from F1.*Scn1a*$^{R1648H/+}$;Q54 and F1.*Scn1a*$^{R1648H/+}$;*Kcnq2*$^{V182M/+}$ offspring and controls for Ohtahara characteristics. Additionally, brain histology of offspring may also support F1.*Scn1a*$^{R1648H/+}$;Q54 and F1.*Scn1a*$^{R1648H/+}$;*Kcnq2*$^{V182M/+}$ offspring as Ohtahara models.

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