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Characterizing the role of sodium channels in mouse models of Dravet Syndrome

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Abstract

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Voltage-gated sodium channels (Na_v) are responsible for the initiation and propagation of action potentials in excitable cells. The channels isoforms $\text{Na}_v1.1$, $\text{Na}_v1.2$, $\text{Na}_v1.3$ and $\text{Na}_v1.6$ are dynamically expressed in the developing central nervous system and are essential for proper network function. Heterozygous loss-of-function mutations in *SCN1A*, the gene encoding $\text{Na}_v1.1$, leads to Dravet Syndrome (DS), a pharmaco-resistant infantile-onset epilepsy syndrome with co-morbidities of cognitive impairment and premature death. Previous studies using a mouse model of DS heterozygous for a global deletion of *Scn1a* (*Scn1a*^{+/-}), revealed reduced sodium currents and impaired excitability in GABAergic interneurons. We generated a floxed *Scn1a* mouse line and used the Cre-Lox method driven by an enhancer from the *DLX1,2* locus to conditionally delete one or both copies of *Scn1a* in forebrain GABAergic neurons. Mice with this specific deletion had selective loss of $\text{Na}_v1.1$ channels in GABAergic interneurons of the cerebral cortex and hippocampus, died

prematurely following generalized tonic-clonic seizures, were equally susceptible to thermal induction of seizures as global *Scn1a*^{+/-} mice, and demonstrated impaired cognitive function. Evidently, loss of Na_v1.1 channels in forebrain GABAergic neurons is sufficient to cause epilepsy, premature death, and cognitive impairments in DS.

Initial characterization of the DS mouse revealed a striking strain difference with respect to survival and seizure susceptibility. Studies also found an interneuron-specific increase in Na_v1.3 with complete loss of Na_v1.1, suggesting Na_v1.3 as a possible precipitating factor or genetic modifier in DS. To evaluate the role of Na_v1.3 in DS progression, we measured channel expression in non-epileptic mouse and human cortical tissue. We found Na_v1.3 was expressed at high levels in embryonic life and declined after birth coincident with increased Na_v1.1 channel expression in both species. The onset of seizures in mouse and human follows the developmental decrease in Na_v1.3 to less than half of its maximal level suggesting that its loss, coupled with failure of normal expression of Na_v1.1 channels, may contribute to the time of onset of seizures in DS. We tested whether genetic deletion of Na_v1.3 channels would exacerbate the early phase of DS in mice and found that heterozygous loss of Na_v1.3 does not lead to impaired survival or increased sensitivity to thermally induced seizures in DS mice. Our results support the hypothesis that declining expression of Na_v1.3 channels to below 50% of maximum, in the face of heterozygous loss-of-function mutation of the Na_v1.1 channel, may be one of the precipitating factors contributing to the time of onset of DS.

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Glossary

CD1: CD1 background mouse line (Jackson Labs)

DS: Dravet Syndrome

EEG: Electroencephalogram

F/F: homozygous floxed $\text{Na}_v1.1$ mutant mouse

F/+ : heterozygous floxed $\text{Na}_v1.1$ mutant mouse

GABA: γ -aminobutyric acid

GAD67: Glutamate Decarboxylase, essential for the synthesis of GABA

GEFS+: Generalized Epilepsy with Febrile Seizures Plus

GTC: Generalized Tonic-Clonic Seizure

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Dedication

I would like to dedicate this thesis to my late grandfather Charles D. Metcalf. He was a man that encouraged me to never stop learning. I wish that he was here today so see the conclusion of my academic journey.

Chapter 1 - Introduction: From Ancient Babylon to the Interneuron

A Brief History of Epilepsy

The earliest known account of epilepsy was found on a Babylonian tablet from 1067-1046 BC. Its authors recorded detailed descriptions of convulsions and attributed these attacks to supernatural or demonic possessions (Reynolds and Kinnier Wilson, 2008). The ancient Chinese and Persian cultures also kept detailed records of convulsions and seizures in their medical texts, as well as a variety of remedies, most considered crude and often ludicrous by today's standards (Eadie, 1995; Chaudhary et al., 2011). By 400 BC, a Hippocratic text on the "*Sacred Disease*" suggested that epilepsy was not caused by spiritual possession, but was instead the result of excess phlegm in the brain. Hippocrates eschewed magical treatments and instead felt that treatment should begin early and focus on the body (Chaudhary et al., 2011).

Although Hippocrates and Galen believed that the brain was responsible for convulsions as early as 400 BC, the brain was not accepted as the source of seizures until the 17th or 18th century. At that time popular views of the cause of epilepsy began to shift from phlegm and animal spirits, to alterations or energetic explosions in the brain (Eadie, 1995; Reynolds and Trimble, 2009; Chaudhary et al., 2011). By the late 19th century John Hughlings Jackson presented an electrical view of dysfunction in the brain and defined epilepsy as "occasional,

sudden, excessive, rapid, and local discharges of gray matter” (Chaudhary et al., 2011). In the 20th century all other theories regarding the cause of epilepsy were largely put to rest and focus shifted solely to understanding the role of electrical activity in the brain after the advent of the EEG. Differences in epileptiform discharges became more clearly characterized in the 1940’s and 1950’s, and today the International League Against Epilepsy (ILAE) defines epileptic seizures as “abnormal, excessive or synchronous neural activity in the brain.” The ILAE also defines epilepsy as a predisposition toward epileptic seizures and the associated behavioral consequences (Fisher et al., 2005). In the early 2000’s, sodium channels emerged as one of the many genes involved in a number of seizure disorders (Catterall et al., 2008; Meisler et al., 2010).

Voltage-gated Sodium Channels

Voltage-gated sodium channels (Na_v) are a homologous family of ten voltage-gated ion channels responsible for the rising phase of an action potential. Each channel is composed of a 260 kDa pore-forming alpha subunit and one or two auxiliary beta subunits (33-36kDa). The alpha subunit alone is sufficient for voltage-dependent gating and ion flux, but the associated beta subunit is essential for conferring normal activation and inactivation kinetics (Catterall, 2000; Isom, 2001). The alpha subunit consists of 4 homologous domains, each containing of 6 alpha-helical transmembrane segments (S1-S6). Both N- and C-termini, as well as the large loops that connect the four homologous domains, are located intracellularly and are targets of channel modulation by protein kinases and protein-protein

interactions, with the loop joining domains III and IV forming the inactivation gate of the channel. The S4 segment of each domain contains positively charged lysine and arginine residues and is responsible for voltage sensing, and sodium selectivity is conferred by the interaction of the S5 and S6 segments and the interconnecting P-loop of each domain (Figure 1.1; Catterall, 2000).

The $\text{Na}_v1.1$, $\text{Na}_v1.2$, $\text{Na}_v1.3$ and $\text{Na}_v1.6$ sodium channel isoforms are prominently expressed in the central nervous system. Differences in the functional roles of channel subtypes can be attributed in part to their individual expression patterns, functional activities, and subcellular localization (Catterall, 2000; Cantrell and Catterall, 2001; Vacher et al., 2008). Sodium channels $\text{Na}_v1.1$ and $\text{Na}_v1.3$ are found in the cell soma and proximal dendrites of neurons, while $\text{Na}_v1.2$ is localized primarily in unmyelinated axons, and $\text{Na}_v1.6$ controls action potential initiation and propagation in myelinated axons and is also expressed in cell bodies and dendrites of some neurons (Vacher et al., 2008; Westenbroek et al., 1989, 1992). A similar pattern of channel distribution is found in humans and this diversity in channel expression allows for finer control of network excitability in both species (Catterall, 2000; Cantrell and Catterall, 2001; Vacher et al., 2008).

The expression of brain sodium channels is developmentally regulated with expression beginning during late gestational development. In rat, $\text{Na}_v1.2$ and $\text{Na}_v1.6$ expression continually increases from birth, while $\text{Na}_v1.1$ expression is very low after birth and increases to a steady state level by the third week of life. $\text{Na}_v1.3$ expression is inversely timed with $\text{Na}_v1.1$, with $\text{Na}_v1.3$ expression highest just prior to birth and declining in early

postnatal life to a steady state level below that of Na_v1.1 by the second week. Even at maximum expression both Na_v1.1 and Na_v1.3 are minority channels when compared to the expression levels of Na_v1.2 and Na_v1.6 (Beckh et al., 1989; Felts et al., 1997; Gong et al., 1999). Human sodium channel development progresses in a similar manner; however, human Na_v1.3 levels can remain elevated in some brain regions into adulthood (Whitaker et al., 2001).

Due to their differing cellular and subcellular localizations, dysfunction of any isoform of the sodium channel family leads to distinct pathological conditions including epilepsy, chronic pain, periodic paralysis and cardiac arrhythmia (Lehmann-Horn and Jurkat-Rott, 1999; Waxman et al., 2000; Catterall et al., 2008). Disease-causing mutations have been found in nearly all brain sodium channels, but Na_v1.1 has been most frequently associated with epilepsy, with nearly 700 mutations reported (Fig. 1.2a; Meisler et al., 2010). Mutations in *SCN1A*, the gene encoding Na_v1.1, were first associated with generalized epilepsy with febrile seizures plus (GEFS+) and have since been associated with many epilepsies (Escayg et al., 2000; Catterall et al., 2010; Meisler et al., 2010).

Na_v1.1 and Epilepsy

Sodium channel gene mutations fall on a continuum of phenotypic severity, where the functional nature of the channel mutation influences the disease phenotype (Figure 1.2; Catterall et al., 2010). For instance, a missense mutation leading to a dysfunctional channel

may result in febrile seizures while a nonsense mutation resulting in a non-functional channel is associated with a more devastating generalized epilepsy. Missense mutations that alter biophysical characteristics of the channel may mediate an epilepsy of intermediate severity (Meisler and Kearney, 2005; Catterall et al., 2010).

Heterozygous complete loss of $Na_v1.1$ function leads to a distinct epilepsy phenotype known as Dravet Syndrome (DS, or Severe Myoclonic Epilepsy in Infancy). DS is a pharmaco-resistant genetic epilepsy characterized by onset of generalized febrile seizures during the first year of life with substantial neuropsychiatric comorbidities that can have negative impact on the quality of life for affected individuals. The nature of these comorbidities is sweeping and includes hyperactivity, delayed psychomotor development, sleep disorders, anxiety-like behaviors, speech delays, impaired social interactions and severe cognitive deficits (Claes et al., 2003; Wolff et al., 2006; Brunklaus et al., 2011; Genton et al., 2011). Mutations are often *de novo*; however, it has also been demonstrated that children can inherit a mutant *SCN1A* from mildly affected or unaffected parents suggesting the importance of genetic background on disease prognosis (Guerrini et al., 2010; Suls et al., 2010).

Mouse models of DS

The effects of the haploinsufficiency of $Na_v1.1$ channels seen in SMEI have been examined utilizing heterozygous $Na_v1.1$ knockout mice or knock-in mutations in our lab and

others (Yu et al., 2006; Ogiwara et al., 2007). Mice heterozygous for a global deletion of Exon-26 of the *Scn1a* gene (*Scn1a*^{+/-}) are an accurate genocopy and phenocopy of DS. These animals develop thermally induced and spontaneous seizures, premature death, and the comorbidities of ataxia and sleep disorders (Yu et al., 2006; Kalume et al., 2007; Oakley et al., 2009; Catterall et al., 2010; Han et al., 2012). Initial characterization of these *Scn1a* mutant animals on a hybrid 129SvJ:C5BL/6 background showed that all knockout (*Scn1a*^{-/-}) animals died at 2 weeks of age (P15), while less than 50% of the heterozygous (*Scn1a*^{+/-}) animals died prematurely beginning in their 3rd week (Fig 1.3a). There were very few deaths in wildtype (WT) mice during this time. However, after successive generations of backcrossing onto the pure C57Bl/6 and 129SvJ strains, differences in survival emerged as C57Bl/6 *Scn1a*^{+/-} animals proved more susceptible to premature death than their 129SvJ genotype correlates (Yu et al., 2006).

As the same *Scn1a* gene ablation was carried by both strains of heterozygous animals, differences in rates of survival between the two mouse strains strongly suggested genetic background influences its survival. In *Scn1a*^{+/-} mice on the hybrid 129SvJ:C5BL/6 background, ICC analysis of brain regions showed a specific increase in Na_v1.3 staining in GAD67 positive interneurons in the hippocampus, suggesting Na_v1.3 as a candidate seizure modifier gene (Figure 1.3b; Yu et al., 2006).

In whole-cell voltage clamp recordings of dissociated hippocampal neurons from WT, *Scn1a*^{+/-} and *Scn1a*^{-/-} animals on a 50:50 C57Bl/6:129SvJ background, a reduction in peak sodium current was only seen in inhibitory interneurons from *Scn1a*^{+/-} and *Scn1a*^{-/-} animals

(Figure 1.3c). Additionally, residual sodium current was seen in inhibitory interneurons from *Scn1a*^{-/-} animals, which suggested another sodium channel may be compensating for the loss of Na_v1.1. Peak sodium current recorded from excitatory pyramidal cells from the same animals remained unchanged among the three genotypes (Figure 1.3c). Functionally, the response to sustained current injection was markedly decreased in the hippocampal interneurons of *Scn1a*^{+/-} and *Scn1a*^{-/-} animals compared to WT (Yu et al., 2006).

Other mouse models have also implicated interneurons as the mediators of loss of Na_v1.1 function. A mouse model homozygous for the human GEFS+ mutation R1648H showed spontaneous generalized seizures and premature death. Heterozygous R1648H mice had infrequent spontaneous generalized seizures and a reduced threshold to febrile seizure. Recordings from GABAergic inhibitory interneurons in this model showed slower recovery from inactivation and reduced action potential firing, while pyramidal cell currents remained unchanged (Martin et al., 2010a). Similarly, a Dravet mouse model utilizing a human DS mutation with a premature stop codon, R1407X, has decreased Na_v1.1 expression and spontaneous seizure activity in R1407X homozygous and heterozygous animals. Staining in WT animals found Na_v1.1 localized to the axon initial segment of Parvalbumin expressing interneurons, and in electrophysiological parvalbumin positive inhibitory cells demonstrated decreased firing following sustained stimulation (Ogiwara et al., 2007). All three of these models implicate interneurons as an important effector of the DS phenotype.

GABAergic Interneurons

To maintain normal network function within the brain, excitation and inhibition exist in balance, and when this balance is disrupted pathological states can emerge (Marin, 2012). Approximately 70-80% of neurons in the neocortex are excitatory glutamatergic pyramidal neurons and the remaining 20-30% are GABAergic inhibitory interneurons (Markram et al., 2004). Although comprising the minority of cells in the brain, interneurons are very diverse in terms of their morphology, connectivity, neurochemistry and electrophysiology (Markram et al., 2004; Wonders and Anderson, 2006). Attempts at classification often center on Parvalbumin, Calbindin, and Calretinin. These three calcium binding proteins are expressed in most GABAergic interneurons, but this method of classification does not account for the individual functional characteristics of the cells. Even within a subclass of interneurons defined by calcium binding proteins, cells do not have identical firing properties or axonal targets. The wide variety of interneurons and their targets points to the importance and complexity of maintaining a balance of excitatory and inhibitory control (Markram et al., 2004; Ascoli et al., 2008).

While classification remains complex, interneuron development is tightly regulated. Most interneurons are born in either the caudal or medial ganglionic eminence and migrate tangentially to their final destination in the neocortex. This maturation and migration process hinges on the proper expression of a number of transcription factors including *Nkx2.1* and the *Dlx* family of homeobox genes (Wonders and Anderson, 2006; Long et al., 2009). Deletion of both *Dlx1* and *Dlx2* in mice results in the failure of interneurons to

migrate out of the ganglionic eminences and a nearly complete loss of cortical interneurons that was incompatible with survival (Anderson et al., 1997). However, when only *Dlx1* is deleted, interneurons were capable of migrating into the cortex but failed to thrive once there. The result of *Dlx1* deletion was a marked decrease of calretinin and somatostatin positive interneurons, decreased GABAergic signaling in the neocortex, and generalized electrographic seizures (Cobos et al., 2005).

Epilepsy is a complex disorder and both sodium channels and interneurons have been implicated in disease pathogenesis. The focus of this thesis is to understand the role $\text{Na}_v1.1$ channel loss in interneuron function and exploring the roles of $\text{Na}_v1.1$ and $\text{Na}_v1.3$ channels during the onset of DS. These two concepts may be beneficial to understanding how the epileptic brain functions and developing new therapeutics in the future.

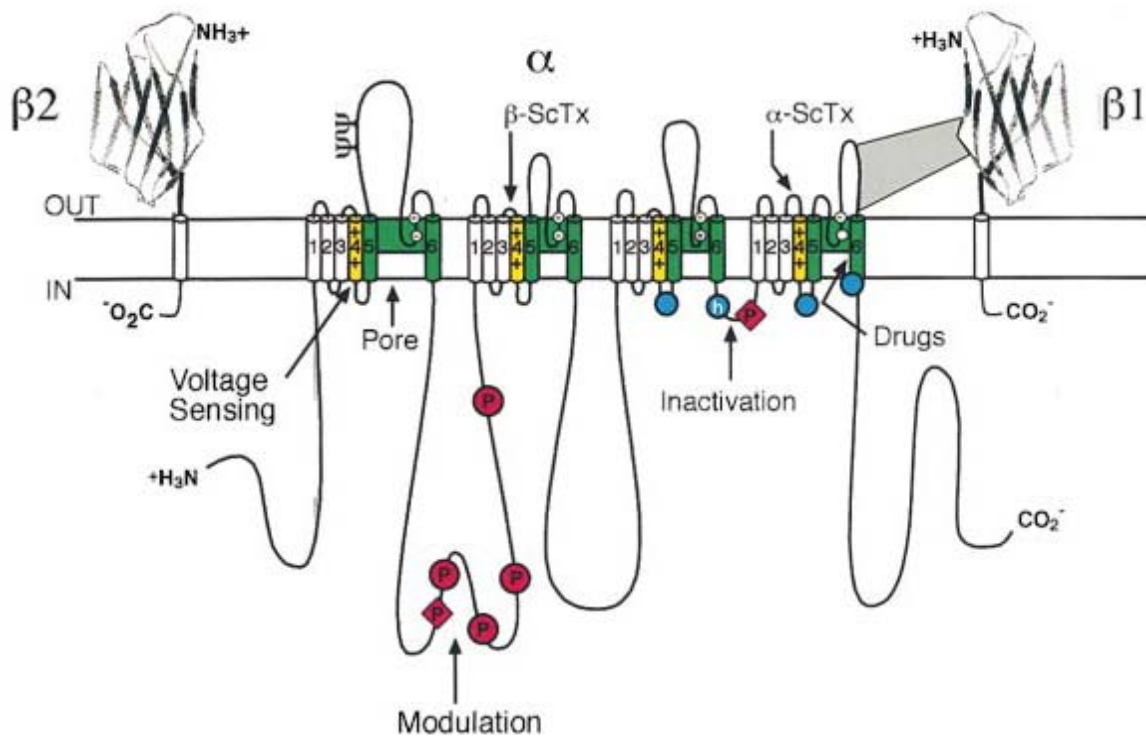


Figure 1.1 Structural schematic of the sodium channel. Cylinders represent α -helical segments, bold lines represent the polypeptide chains of each subunit with length approximately proportional to the number of amino acid residues in the brain sodium channel subtypes. The extracellular domains of the $\beta 1$ and $\beta 2$ subunits are shown as immunoglobulin-like folds. P in red circles, sites of demonstrated protein phosphorylation by PKA (circles) and PKC (diamonds); yellow, S4 voltage sensors; h in blue circle, inactivation particle in the inactivation gate loop; blue circles, sites implicated in forming the inactivation gate receptor (Catterall, 2000).

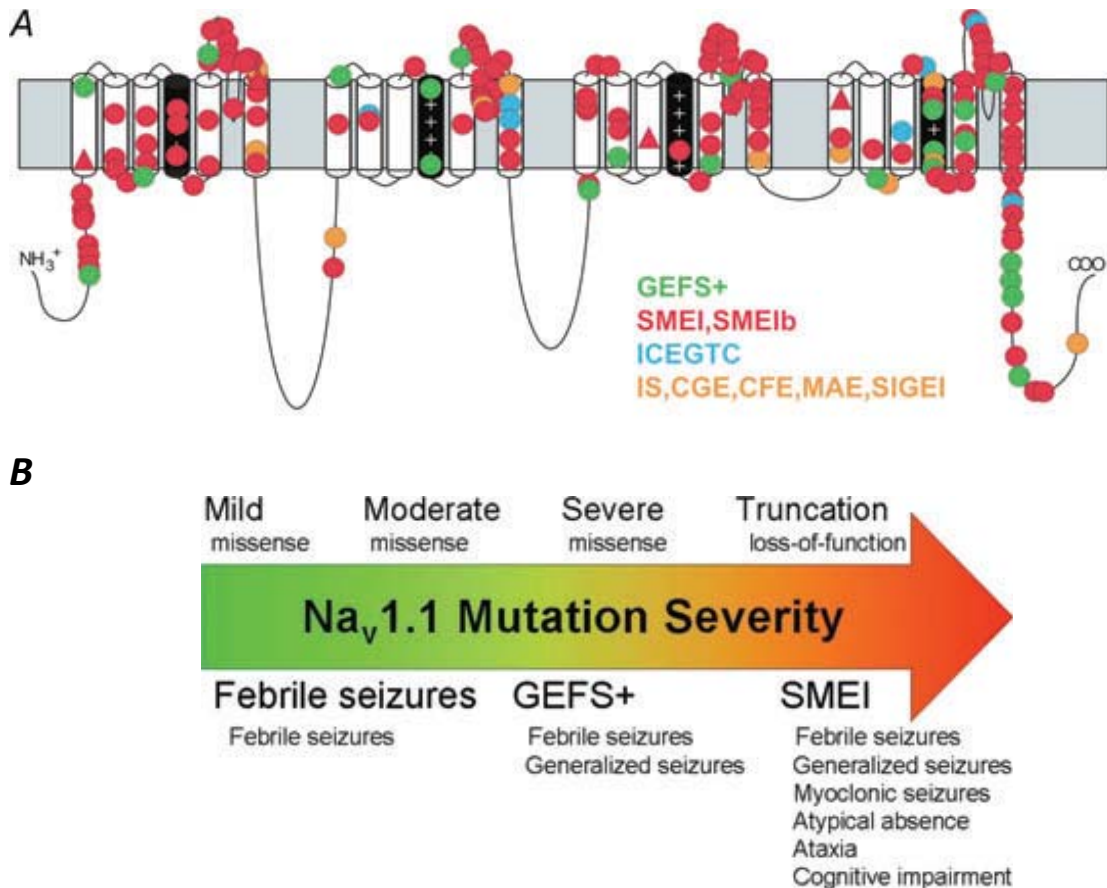
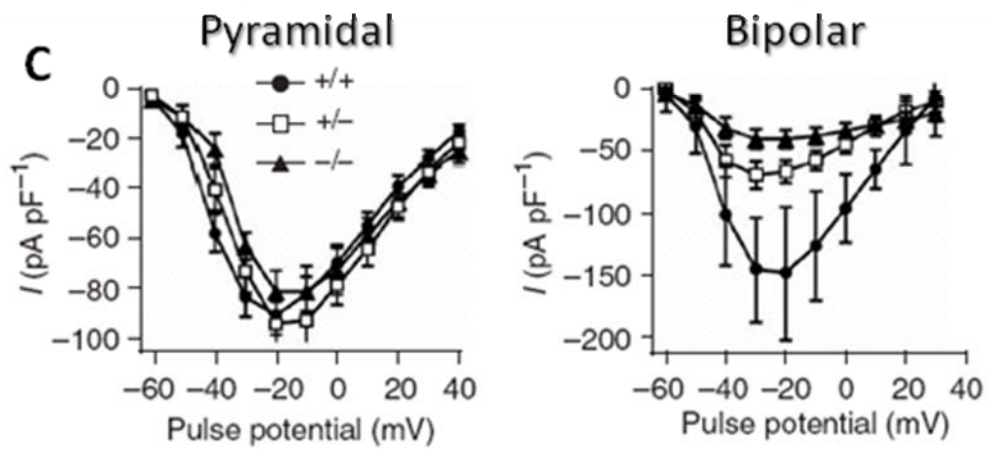
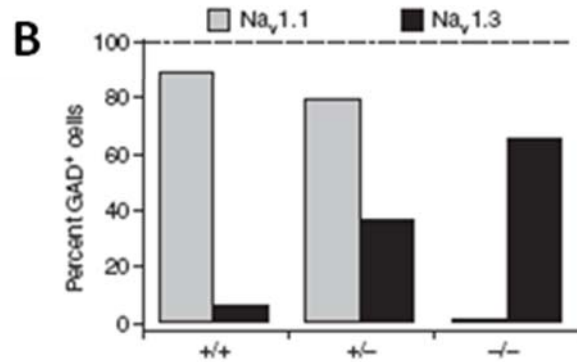
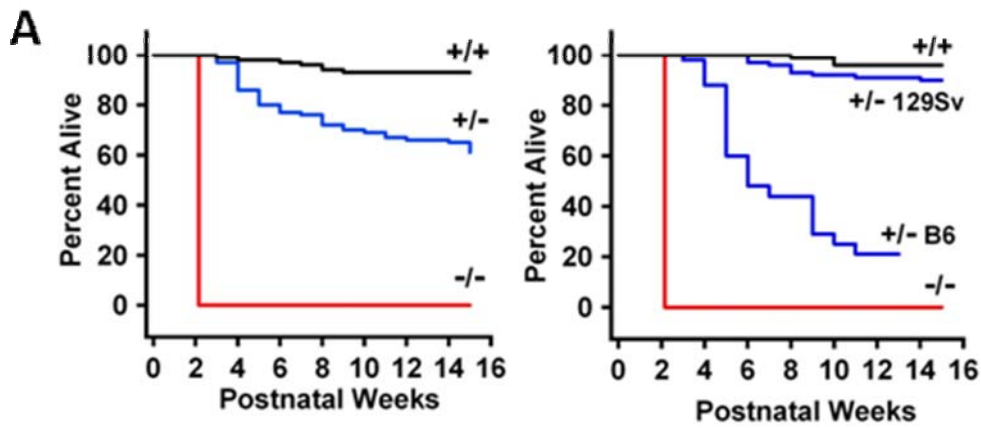


Figure 1.2 Continuum of sodium channel mutations and severity of epilepsy. (A) Non-truncating epilepsy causing mutations represented on the channel schematic. Missense mutations (circles) and in-frame deletions (triangles). The clinical type of epilepsy is indicated by color: GEFS+, generalized epilepsy with febrile seizures plus; SMEI (DS), severe myoclonic epilepsy of infancy (Dravet Syndrome); SMEIb, borderline SMEI; ICEGTC, idiopathic childhood epilepsy with generalized tonic-clonic seizures; IS, infantile spasms; CGE, cryptogenic generalized epilepsy; CFE, cryptogenic focal epilepsy; MAE, myoclonic astatic epilepsy; SIGEI, severe idiopathic generalized epilepsy of infancy (Catterall et al., 2008). (B) The increasing severity of sodium channel mutations (noted above the arrow) and associated epilepsy syndromes (noted below the arrow) demonstrate the progressive increase in severity of epilepsy syndromes from familial febrile seizures to GEFS+ and finally SMEI (DS). Major symptoms of each syndrome are also listed (Catterall et al., 2010).

Figure 1.3 DS phenotype in global $Scn1a^{+/-}$ mice. (A) Premature deaths of $Na_v1.1$ mutant mice on 129SvJ:C57BL/6 genetic background and 129SvJ and C57BL/6 genetic backgrounds. *Left*, Survival plot of WT, $Scn1a^{+/-}$, and $Scn1a^{-/-}$ mice on the hybrid 129SvJ:C57BL/6 background as a percentage of live mice at each postnatal week of age (WT, n=233; $Scn1a^{+/-}$, n= 277; $Scn1a^{-/-}$, n = 75). *Right*, Survival plot of WT, $Scn1a^{+/-}$, and $Scn1a^{-/-}$ mice on 129SvJ and C57BL/6 genetic backgrounds as a percentage of live mice at each postnatal week of age (WT 129Sv, n=107; $Scn1a^{-/-}$ 129SvJ, n=34; $Scn1a^{+/-}$ 129SvJ, n=111; $Scn1a^{+/-}$ C57BL/6, n=52) (B) $Na_v1.3$ upregulation in 129SvJ:C57BL/6 $Na_v1.1$ mutant mice. Bar graph illustrating the percentage of GAD-positive interneurons in the hippocampus that were colabeled with either anti- $Na_v1.1$ or anti- $Na_v1.3$ in WT, $Scn1a^{+/-}$ or $Scn1a^{-/-}$ mice. (C) Current density–voltage relationships of whole-cell sodium currents from hippocampal pyramidal (*left*) and bipolar (*right*) neurons for WT, $Scn1a^{+/-}$ and $Scn1a^{-/-}$ mice. Currents of $Scn1a^{+/-}$ and $Scn1a^{-/-}$ bipolar neurons demonstrate a non-linear reduction of peak sodium current ($P < 0.05$). (Yu et al., 2006)



Chapter 2 - Specific Deletion of Na_v1.1 Channels in Inhibitory

Interneurons Causes Seizures and Premature Death in a Mouse

Model of Dravet Syndrome

This chapter is in press at PNAS as: “Cheah CS, Yu FH, Westenbroek RE, Kalume FK, Oakley JC, Potter GB, Rubenstein JL, Catterall WA. Specific Deletion of Na_v1.1 Channels in Inhibitory Interneurons Causes Seizures and Premature Death in a Mouse Model of Dravet Syndrome. (2012)”

Abstract

Heterozygous loss-of-function mutations in the brain sodium channel Na_v1.1 cause Dravet Syndrome (DS), a pharmaco-resistant infantile-onset epilepsy syndrome with co-morbidities of cognitive impairment and premature death. Previous studies using a mouse model of DS revealed reduced sodium currents and impaired excitability in GABAergic interneurons in the hippocampus, leading to the hypothesis that impaired excitability of GABAergic inhibitory neurons is the cause of epilepsy and premature death in DS. However, other classes of GABAergic interneurons are less impaired, so the direct cause of hyperexcitability, epilepsy, and premature death has remained unresolved. We generated a floxed *Scn1a* mouse line and used the Cre-Lox method driven by an enhancer from the *DLX1,2* locus for conditional deletion of *Scn1a* in forebrain GABAergic neurons. Immunocytochemical studies demonstrated selective loss of Na_v1.1 channels in GABAergic interneurons in cerebral

cortex and hippocampus. Mice with this deletion died prematurely following generalized tonic-clonic seizures, and they were equally susceptible to thermal induction of seizures as mice with global deletion of *Scn1a*. Evidently, loss of Na_v1.1 channels in forebrain GABAergic neurons is both necessary and sufficient to cause epilepsy and premature death in DS.

Introduction

Voltage gated sodium (Na_v) channels are composed of a 260 kDa pore-forming α subunit and one or more smaller auxiliary β subunits (Catterall, 2000, Isom, 2001). The Na_v1.1, Na_v1.2, Na_v1.3, and Na_v1.6 isoforms are highly expressed in the brain, where they initiate and propagate action potentials in neurons. Na_v1.1 and Na_v1.3 are prominently expressed in the cell soma and axon initial segment where they integrate incoming information from the dendrites, whereas Na_v1.2 channels are found in unmyelinated axons and dendrites, and Na_v1.6 channels are found in cell bodies, dendrites, and the nodes of Ranvier of myelinated axons (Westenbroek et al., 1989; Gong et al., 1999; Catterall, 2000; Trimmer and Rhodes, 2004; Yu and Catterall, 2004). While each channel is responsible for sodium conduction through the membrane, their differential localization leads to discrete disease pathologies (Catterall et al., 2008). Mutations in the pore-forming subunits of Na_v1.1 channels cause multiple types of epilepsy (Catterall et al., 2010, Meisler et al., 2010). Heterozygous missense and truncation mutations in the SCN1A gene encoding Na_v1.1 channels lead to haploinsufficiency of the channel function and cause Dravet Syndrome (DS,

also known as Severe Myoclonic Epilepsy of Infancy; Claes et al., 2001; Sugawara et al., 2003; Bechi et al., 2012). This disease begins with infantile-onset febrile seizures at 6-9 months of age and progresses to intractable afebrile, generalized seizures in later years. In addition to seizures, patients with DS suffer from several severe co-morbidities including ataxia, psychomotor delay, cognitive impairment, and often premature death (Dravet, 2011).

In mice, heterozygous deletion of exon-26 in *Scn1a* leads to global haploinsufficiency of Na_v1.1 and recapitulates the symptoms of DS (Yu et al., 2006). These global heterozygous mice exhibit temperature-induced and spontaneous seizures, mild ataxia, and premature death (Yu et al., 2006; Kalume et al., 2007; Oakley et al., 2009). Na_v1.1 channels are expressed in both excitatory and inhibitory neurons (Westenbroek et al., 1989, Trimmer and Rhodes, 2004), yet electrophysiological studies in dissociated hippocampal neurons from DS mice showed selective loss of sodium current and excitability in GABAergic interneurons (Yu et al., 2006, Ogiwara et al., 2007). These results led to the hypothesis that the epilepsy and co-morbidities of DS are caused by selective impairment of inhibitory interneuron function. However, subsequent studies have revealed lesser impairments of sodium current and excitability in GABAergic inhibitory neurons in the cerebellum, reticular nucleus of the thalamus, and cerebral cortex (Kalume et al., 2007, 2010; Abe et al., 2010), so this hypothesis requires further validation.

To target gene expression in GABAergic neurons selectively, we have taken advantage of the *Dlx* gene family, which encodes homeobox transcription factors that are specifically

required for specification, maturation, and survival of forebrain GABAergic neurons (Wonders and Anderson, 2006, Long et al., 2009). Deletion of one of these genes, *Dlx1*, in mice leads to selective interneuron loss and seizures (Cobos et al., 2005). In the *Dlx1/2-112b-Cre* transgenic mouse, an intergenic *Dlx1* and *Dlx2* enhancer drives expression of Cre recombinase specifically in GABAergic neurons in the forebrain (Potter et al., 2009). We have used this *Dlx-Cre* mouse line in conjunction with a floxed $\text{Na}_v1.1$ mouse line to directly test the hypothesis that deletion of $\text{Na}_v1.1$ channels in the forebrain is sufficient to recapitulate the premature death and seizure phenotypes observed in the $\text{Na}_v1.1$ -deletion model of DS.

Results

Generation of a Floxed $\text{Na}_v1.1$ Mouse Line.

Homologous recombination was used to replace exon-25 of the endogenous *Scn1a* gene using a targeting vector containing the exon flanked by LoxP sites and a neomycin-selection cassette, which itself was flanked by FRT sites (Fig. 2.1A). Prior to breeding animals for experiments, the neomycin-selection cassette was removed to avoid possible impairment of *Scn1a* function by mating a homozygous flox (F/F) mouse with a flipase-expressing mouse. This genetic cross excises the neomycin selection cassette and leaves only a single FRT site (Fig. 2.1A). Flox heterozygous (F/+) and homozygous (F/F) mice maintained on a C57BL/6 background are indistinguishable from WT littermates and breed, thrive and survive

normally. Breeding with a globally-deleting Meox2-Cre strain (Jackson Labs) excised exon-25 and resulted in the expected truncated PCR product (Fig. 2.1B) in DNA samples from F/+;Meox2-Cre⁺ and F/F;Meox2-Cre⁺ mice at postnatal day (P) 14. Immunoblotting of protein samples from these mice showed a substantial reduction in Na_v1.1 protein levels in F/+;Meox2-Cre⁺ mice and no detectable Na_v1.1 protein in F/F;Meox2-Cre⁺ mice at postnatal day P14 (Fig. 2.1C). Immunocytochemical studies with specific antibodies against Na_v1.1 protein revealed a visually evident reduction in staining of the cell bodies of GABAergic inhibitory neurons in layers IV and VI in somatosensory cortex, which contain primarily interneurons, whereas there was no evident decrease in immunostaining of neuronal cell bodies in layer V, where the cell bodies of excitatory pyramidal neurons are located (Fig. 2.1D). Further quantitative analysis of the selective deletion of Na_v1.1 channels is presented below.

Specific Deletion of Na_v1.1 Protein in GABAergic Neurons in Dlx-112b-Cre Mice.

The *Dlx* gene family encodes homeobox transcription factors that are essential for specification, maturation, and survival of interneuron progenitors ((Wonders and Anderson, 2006)). Mating with the Dlx1/2-112b-Cre (Dlx-Cre; Potter et al., 2009) mouse targets Cre recombinase expression to forebrain interneurons, and conditional deletion of *Dlx* leads to interneuron loss and seizures (Cobos et al., 2005). Our C57BL/6 F/F mice were crossed with

Dlx1/2-112b-Cre⁺ (Dlx-Cre⁺) mice to produce heterozygous loss of Na_v1.1 channels in interneurons of the cerebral cortex and hippocampus on a C57BL/6:CD1 background. This genetic cross was consistent through all experiments and all comparisons were made between Dlx-Cre⁻ and Dlx-Cre⁺ littermates.

Double-labeling with antibodies recognizing Na_v1.1 and GABA revealed a specific decrease in immunostaining for Na_v1.1 in GABA-expressing inhibitory interneurons, as illustrated in the dentate gyrus from F/F:Dlx-Cre⁺ and F/F:Dlx-Cre⁻ mice (Fig. 2.2A-F). The arrowheads indicate individual examples of neurons that are double-labeled in F/F:Dlx-Cre⁻ mice (Fig. 2.2A-C) but only labeled with GABA in F/F:Dlx-Cre⁺ mice (Fig. 2.2D-F). Arrows indicate clusters of 2-4 neurons that are double-labeled in F/F:Dlx-Cre⁻ mice but not in F/F:Dlx-Cre⁺ mice (Fig. 2.2A-F). Similar images were obtained for the visual and somatosensory cortex (Fig. 2.5). Quantification of this double-labeled immunostaining showed a significant reduction in the fraction of Na_v1.1/GABA double-labeled interneurons in F/F:Dlx-Cre⁺ brain sections compared to F/F:Dlx-Cre⁻ controls across several regions of the cerebral cortex and hippocampus (Fig. 2.2G). As expected, F/F:Dlx-Cre⁺ brain slices retained normal numbers of Na_v1.1-expressing layer V pyramidal cells in visual cortex and somatosensory cortex at P14 when compared to F/F:Dlx-Cre⁻ slices (Fig. 2.2H). GABA-positive neurons do not all contain Na_v1.1, and Cre expression is not expected to result in complete loss of Na_v1.1 expression in all cells because of differences in timing and extent of activation by the *Dlx* enhancer. We determined the average pixel intensity of Na_v1.1 staining in GABA-positive and GABA-negative staining cells and observed that the intensity of immunostaining of Na_v1.1 in

remaining Na_v1.1/GABA double-labeled interneurons in F/F:Dlx-Cre⁺ mice was reduced compared to F/F:Dlx-Cre⁻ littermates , but no change in Na_v1.1 staining intensity was observed in Na_v1.1-positive, GABA-negative, excitatory pyramidal cells in layer V of the cerebral cortex or in dentate granule cells (Fig. 2I). These results demonstrate selective deletion of Na_v1.1 channels in GABAergic interneurons in the hippocampus and cerebral cortex.

Heterozygous Conditional Deletion of Na_v1.1 Leads to Premature Death Following Spontaneous Seizures.

F/+Dlx-Cre⁺ mice died prematurely compared to F/+Dlx-Cre⁻ littermates, with initial deaths beginning late in the third week and only 30% of animals remaining at 90 days (Fig. 2.3A). Video recordings in a separate population of animals captured spontaneous seizures in approximately one-third of the F/+Dlx-Cre⁺ mice, which were scored 1-5 using the Racine Scale (n=10/35; Racine, 1972). Seizures and death in these animals followed a stereotyped progression. Seizures began with forelimb clonus (Racine 3) and progressed in both frequency and severity through forelimb clonus with rearing (Racine 4), eventually culminating in generalized tonic-clonic seizure (GTC, Racine 5), and finally death 4-48 h after initial seizure onset. This time course of seizures and death is illustrated for a typical animal in Fig. 2.3B. Age at death ranged from P18-P22 and all deaths were observed immediately following a Racine 5 GTC seizure (Fig. 2.3B, C). Animals died an average of 17.9 h after seizure onset, during which time they experienced an average of 9 seizures (Fig. 2.3D, E).

This timing of death in the population of mice monitored for spontaneous seizures correlated with the time of highest incidence of death in the total population, suggesting seizures precede death in the general population. Throughout this time period, no F/+;Dlx-Cre⁻ animals were observed to have seizures or die (n=28). These results show that the premature death observed in F/+;Dlx-Cre⁺ mice is comparable to that in Na_v1.1 global heterozygotes (Yu et al., 2006) and therefore provide support for the conclusion that heterozygous deletion of Na_v1.1 channels in forebrain GABAergic inhibitory neurons is sufficient to cause premature death in the mouse model of DS.

Heterozygous Conditional Deletion of Na_v1.1 Leads to Thermal Sensitivity to Seizure.

Patients with DS (Dravet, 2011) and our mouse model of this disease (Oakley et al., 2009) are susceptible to seizures evoked by elevation of body temperature. When the core body temperature of our F/+;Dlx-Cre⁺ animals was increased, seizures were induced in all animals tested with mean temperature for seizure induction of 39.6°C (Fig. 2.4A; F/+;Dlx-Cre⁺, n=17). Conversely, no F/+;Dlx-Cre⁻ animals exhibited thermally induced seizures (Fig. 2.4A; F/+;Dlx-Cre⁻, n=10). Seizures provoked by thermal induction ranged in severity from simple forelimb clonus (Racine 3) to full GTC (Racine 5), but the majority of animals had Racine 4 seizures (Fig. 2.4B). The two animals that progressed to GTC died soon thereafter. These results show that the sensitivity to thermal induction of seizures in our F/+;Dlx-Cre⁺ is indistinguishable from our global Na_v1.1 heterozygotes, supporting the conclusion that heterozygous deletion of Na_v1.1 channels in forebrain GABAergic inhibitory neurons is

sufficient to cause the susceptibility to thermally induced seizures observed in our mouse model of DS.

A subset of animals were implanted with four EEG leads at P24, and EEG's were recorded during thermal induction on the following day after the mice recovered from surgery and were free from the effects of anesthesia. In each F/+;Dlx-Cre+ animal tested, electrographic seizures and behavioral correlates were closely linked (F/+;Dlx-Cre+, n=4, Fig.2. 4C). In contrast, none of the F/+;Dlx-Cre- mice experienced either electrographic seizures or behavioral correlates (F/+;Dlx-Cre-, n=5, Fig.2. 4C). The pattern of epileptiform electrical activity preceding and during the seizures induced in F/+;Dlx-Cre+ mice were similar to those characterized previously in global Na_v1.1 heterozygotes (Yu et al., 2006; Oakley et al., 2009) and in children with DS (Oguni et al., 2001).

Lack of Effect of Heterozygous Conditional Deletion of Na_v1.1 in the Heart.

Na_v1.1 channels are expressed in the mouse heart (Maier et al., 2003); therefore, it is possible that a direct effect of our gene deletion on cardiac function could contribute to seizures or premature death. In an extensive study using sensitive detection methods, Potter et al. demonstrated that Dlx-12b-cre expression is restricted to the forebrain and no expression was observed in the peripheral nervous system or heart (Potter et al., 2009). Our findings that deletion of Na_v1.1 channels is specific to inhibitory neurons and not excitatory neurons further demonstrates the specificity of this gene deletion and indicates that no

leaky Cre expression has occurred in our F/+;Dlx-Cre+ mice. Consistent with these studies, ECG recordings at rest revealed no significant differences between F/+;Dlx-Cre- (n=5) and F/+;Dlx-Cre+ (n=6) animals in heart rate (F/+;Dlx-Cre-, 672.2 ± 24 ; F/+;Dlx-Cre+, 658.4 ± 57), PR interval (F/+;Dlx-Cre-, 32.8 ± 2 ; F/+;Dlx-Cre+, 32.3 ± 2), QRS interval (F/+;Dlx-Cre-, 9.4 ± 1 ; F/+;Dlx-Cre+, 8.3 ± 1) or QT interval (F/+;Dlx-Cre-, 23.5 ± 5.5 ; F/+;Dlx-Cre+, 19.7 ± 1.5 ; Fig. 2.4D, $p > .05$). These results demonstrate that selective deletion of $Na_v1.1$ channels in forebrain neurons has no effect on intrinsic action potential generation and conduction in the heart and therefore support the conclusion that seizures and premature deaths in our conditional deletion mouse model of DS is initiated by seizures.

Discussion

Dysfunction of GABAergic interneurons is known to produce seizures in mice (Cobos et al., 2005). Moreover, GABAergic interneurons in the hippocampus have a profound and selective loss of sodium current and electrical excitability in global loss-of-function mouse models of DS (Yu et al., 2006, Ogiwara et al., 2007). However, studies of other GABAergic inhibitory neurons in the cerebellum, reticular nucleus of the thalamus, and cerebral cortex have revealed smaller losses of sodium currents and lesser impairments of excitability in our mouse model of DS (Kalume et al., 2007, 2010; Abe et al., 2010). Therefore, these previous observations did not establish a causal relationship between the loss of sodium current and action potential firing in GABAergic interneurons and the hyperexcitability, seizure, and premature death in DS. Our experiments using the Cre-Lox method of conditional gene

deletion clearly demonstrate that loss of Na_v1.1 only in GABAergic inhibitory Dlx1/2-112b-Cre⁺ interneurons in the forebrain replicates the severe spontaneous and temperature-induced GTC seizures observed in a global DS model. Furthermore, our data show that premature death in this animal model is precipitated by spontaneous seizures caused by deletion of Na_v1.1 channels in forebrain inhibitory neurons. These results show directly that hyperexcitability and seizures in mouse DS are caused by loss of GABAergic interneuron excitability.

There are many different classes of interneurons in the forebrain; therefore, our results do not provide insight into the specific classes of forebrain interneurons whose loss of excitability leads to seizures and premature death. In light of the very large loss of sodium current in GABAergic inhibitory neurons in the hippocampus in our mouse model of DS (50% loss of sodium current in dissociated inhibitory neurons in heterozygotes), our working model is that failure of firing of GABAergic interneurons in the hippocampus is one important component of the functional impairment in this disease. In contrast, similar studies of the entire dissociated GABAergic interneuron pool in the cerebral cortex did not reveal a significant loss of sodium current (Abe et al., 2010). These results illustrate a profound difference between the role of Na_v1.1 channels in GABAergic interneurons in the hippocampus and cerebral cortex. The lack of detectable effect of deletion of Na_v1.1 channels on sodium currents measured in whole-cell voltage clamp studies of cortical GABAergic interneurons might indicate that these channels comprise only a small fraction of the sodium current in the cell bodies in the major classes of these neurons. Alternatively,

other Na_v channels may be up-regulated to compensate for the loss of Na_v1.1 channels, or the relevant Na_v1.1 channels in these cells may be located in the axon initial segment and therefore are lost in dissociated cells as prepared for whole-cell voltage clamp. In any case, further studies with more selective gene-knockout methods and more detailed physiological recordings of GABAergic interneurons *in situ* in brain slices will be necessary to identify the specific neuron types in the cerebral cortex whose loss of Na_v1.1 channels contributes to seizures and premature death in our mouse model of DS.

Premature death in our mouse model parallels the high percentage of sudden unexplained death in epilepsy (SUDEP) in DS patients (Sakauchi et al., 2011, Genton et al., 2011). Careful review of our video records showed that premature deaths occur following Racine 5 seizures of short duration (< 2 min), rather than during extended periods of status epilepticus (> 30 min), and therefore would be classified as SUDEP as generally defined in human epilepsy. Na_v1.1 channels are expressed throughout the central nervous system and in the sino-atrial node, atria, and ventricles in the mouse heart (Maier et al., 2002, 2003). Our results show that loss of Na_v1.1 channels in GABAergic interneurons in the forebrain is sufficient to cause premature death that is similar to global heterozygotes. These results eliminate loss of Na_v1.1 channels *per se* in the spinal cord, peripheral nervous system, or heart as a requirement for premature death and show that fatal dysfunction originates with failure of firing of forebrain GABAergic interneurons and the resulting Racine 5 seizures. Further studies in which EEG, ECG, and respiratory recordings are captured during spontaneous seizures and deaths will be required to determine how the seizures caused by

loss of Nav1.1 channels in GABAergic interneurons in the forebrain actually cause premature death, which could result from primary brain damage, respiratory failure, or heart failure caused by seizures.

Materials and Methods

Generation and Maintenance of Floxed Mouse Line

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Washington. Exon-25, the second to last coding exon of *Scn1a*, was cloned into plasmid 4517D (a gift from Dr. G. Stanley McKnight, University of Washington) containing a single LoxP site, FRT-flanked neomycin selection cassette, and multiple cloning sites. The Nhe/Sal sites were used to insert a 6 kb long arm containing exon-26 (DIV S3-CT) and the Xho/Not sites were used to insert a 1.5 kb short arm containing exon-25 (DIV S1-S3) and second LoxP site. The plasmid was linearized at the AscI site, electroporated into embryonic stem cells, and used to generate chimeric mice. Animals were genotyped using FHY311 (5'-CTTGATGTGTTGAAATTCAC-3') and FHY314 (5'-TATAGAGTGTTTAATCTCAAC-3'): WT allele, 846 bp; floxed allele, 1019 bp; excised allele, 258 bp. The neomycin selection cassette was removed following mating with a flipase expressing mouse (B6.SJL-Tg(ACTFLPe)9205Dym/J, Jackson Labs), and the resulting neomycin excised animals were backcrossed with C57BL/6J (Jackson Labs) for 10 generations. *Dlx1*-Cre mice (obtained from Dr. John L. Rubenstein, University of California, San Francisco) were received and

maintained on a CD1 (Jackson Labs) background. Mouse lines were maintained independently and mated together to generate F1, F/+:Dlx-Cre⁺ and F/+:Dlx-Cre⁻ littermates. All experimental comparisons were made between F1 littermates.

Immunohistochemistry

Animals were perfused at P14 with 4% paraformaldehyde in PBS, brains were removed and saturated in 30% sucrose, sliced to 50 μ m, and double-labeled as free-floating slices (Westenbroek et al., 1998). Primary antibodies were affinity-purified rabbit anti-Na_v1.1 (1:150, SP11A; Westenbroek et al., 1989) and guinea pig anti-GABA (1:600, Abcam). Secondary antibodies were goat anti-guinea pig IgG labeled with Alexa555 (1:400, Invitrogen), goat anti-rabbit IgG labeled with Alexa488 (1:400, Invitrogen). Tissue samples from F/F:Dlx-Cre⁻, F/F:Dlx-Cre⁺ and global *Scn1a* knockout animals were processed simultaneously. Gain- and offset-matched images were collected on a Leica SL confocal microscope at the Keck Imaging Facility of the University of Washington. Sections stained in the absence of primary antibody showed no detectable labeling, similar to the global Na_v1.1-knockout tissue stained with Na_v1.1 antibodies.

Spontaneous Seizure Recording and Analysis

Animals were continuously video-monitored from P19-P27. The resulting video files were reviewed at ~8x speed. Suspected seizure events were reviewed at 2x speed and scored

from 1-5 for seizure severity based on the Racine scoring system: 1, mouth and facial movements; 2, head nodding; 3, forelimb clonus, usually one limb; 4, forelimb clonus with rearing; 5, generalized tonic-clonic seizure (GTC), rearing, clonus and falling over (Racine, 1972).

Thermal Induction and EEG

Each animal's core body temperature was continuously monitored by a rectal temperature probe and controlled by a feedback circuit in line with a heat lamp. Body temperature was increased in 0.5°C steps at 2 min intervals until a seizure occurred or a temperature of 42°C was reached. The animal was then cooled and returned to its home cage. Thermal induction was performed on P22, and the induction process and resulting seizures were recorded by video monitoring. Seizures were scored on the Racine Scale using the same criteria as in spontaneous seizure monitoring. EEG surgeries were performed at P23 with the animals under ketamine/xylazine anesthesia and aseptic conditions. Animals were implanted with 4 to 6 small platinum wire electrodes and allowed to recover for 24 hours prior to referential EEG recording during thermal induction (Oakley et al., 2009).

Electrocardiogram Recordings

Surgeries to implant ECG electrodes were performed under ketamine/xylazine (130/8.8 mg/kg) anesthesia and aseptic conditions. A small midline skin incision was made above the

skull and a second incision above the thorax. Two silver electrodes were tunneled subcutaneously from the head incision to the thoracic incision site and anchored in place with suture. One electrode was positioned near the heart apex and the other near the right forelimb. Mice were allowed to recover from surgery overnight. On experiment day, mice were transferred to the recording chamber and allowed to acclimate for 30 min before recording. ECG recordings were collected in conscious mice in daytime on a PowerLab 35 data acquisition unit using LabChart software (ADInstruments, Colorado Spring, Co), at 1 KHz sampling rate and processed off-line with a 3-Hz highpass filter.

Figure 2.1 Floxed Scn1a mouse reduces protein expression in presence of Cre. (A) Schematic representation of the targeting construct used to generate our floxed Scn1a mouse. Exon-25 is flanked on the 5' and 3' ends by LoxP sites in combination with an FRT flanked neomycin selection marker (top panel). The neomycin cassette was removed in breeding cross of F/F animals with a flipase expressing animal, leaving behind a single FRT site (middle panel). In the presence of Cre recombinase exon-25, the FRT site and one LoxP site are excised leaving behind a single LoxP site (bottom panel). (B) Representative genomic DNA gel of PCR products from +/+, F/F and F/+ tail DNA in the absence of Meox2-Cre and F/+;Meox2-Cre+ demonstrating an excision of exon-25 and a corresponding decrease in PCR product size. WT allele: 846 bp, Flox allele: 1009 bp, Excised allele: 258 bp (C) Western blot for Na_v1.1 protein expression in whole brain membrane proteins (150 µg) from P14 F/+;Meox-Cre-, F/+;Meox-Cre+ and F/F;Meox-Cre+ demonstrating a stepwise decline in Na_v1.1 protein levels with the successive removal of exon-25 in the presence of Meox2-Cre. The F/F;Meox2-Cre+ sample contains no Nav1.1 protein and recreates the global knockout expression profile as expected. (D) Following mating a F/F female with a Dlx-Cre+ male a visually detectable decrease in Na_v1.1 immunoreactive staining in deep somatosensory cortex at P14 can be seen. *Left*, F/F;Dlx-Cre-. Immunostaining of neuronal cell bodies for Na_v1.1 channels. *Center*, F/F;Dlx-Cre+. Immunostaining of Na_v1.1 channels in layer V pyramidal cells appears intact, but decreases in immunostaining for Na_v1.1 channels in layer IV and VI cells can be detected. *Right*, global Na_v1.1 knockout, included as a negative control for antibody specificity. Na_v1.1 protein expression is not observed in an age and gain-matched global knockout at P14.

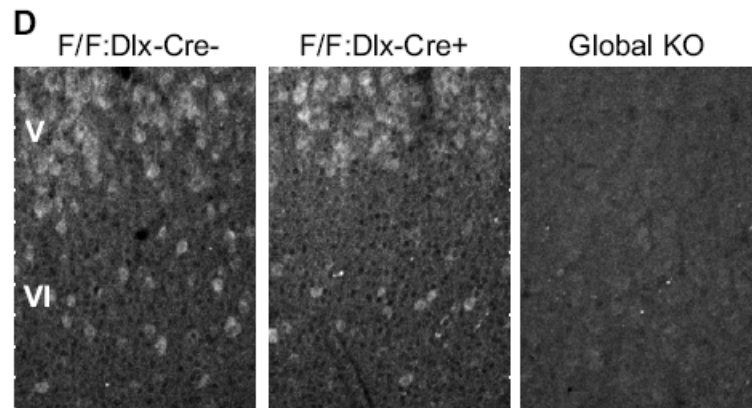
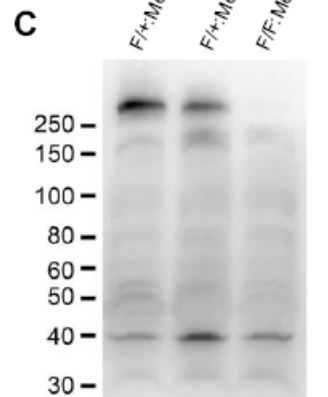
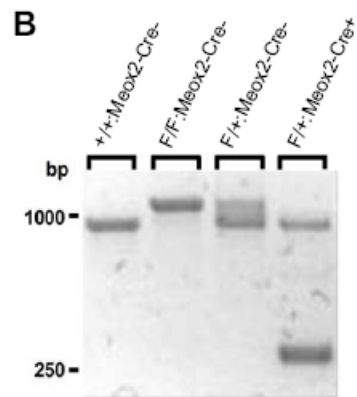
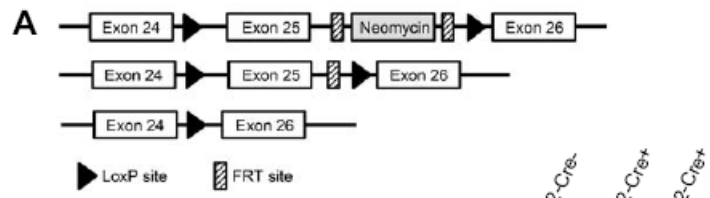
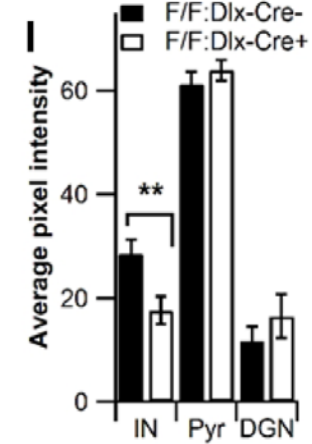
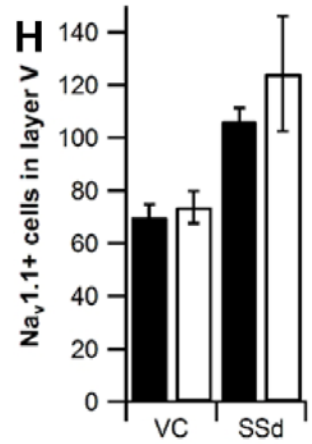
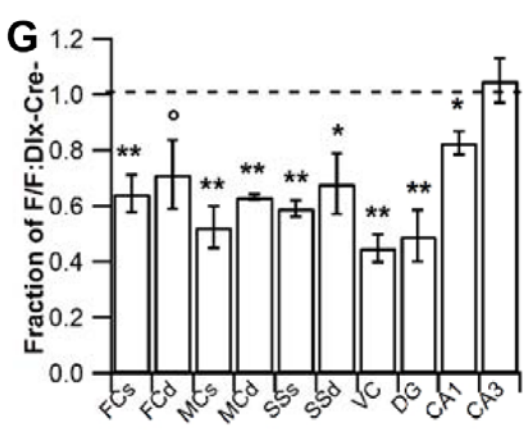
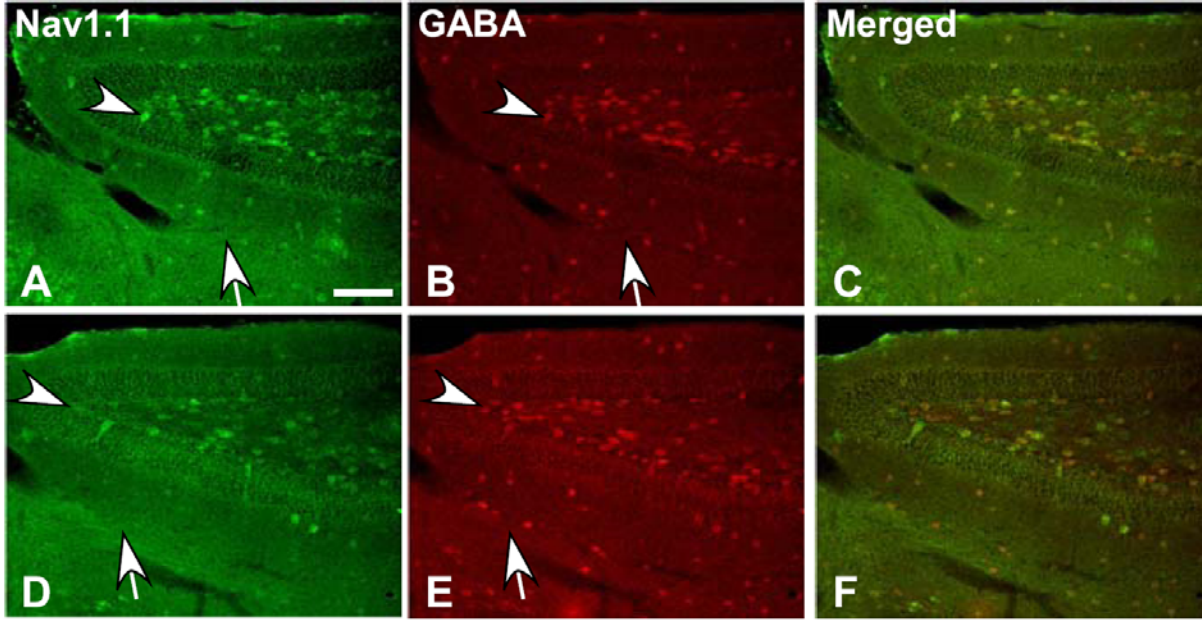


Figure 2.2. Dlx1/2-I12b-Cre preferentially decreases Nav1.1 expression in interneurons. (A-F) Representative immunostaining for Nav_v1.1 (*green*), GABA (*red*) and merged (*yellow*) in the dentate gyrus from F/F:Dlx-Cre⁻ (A-C) and F/F:Dlx-Cre⁺ (D-F) demonstrate a selective loss of cell body Nav_v1.1 staining in F/F:Dlx-Cre⁺ slices in GABA positive cells of the hilus (Arrowheads) and molecular layer (Arrows). (G) Fraction of neurons in F/F:Dlx-Cre⁺ tissue co-immunostained for GABA and Nav_v1.1 channels quantified across a range of brain regions relative to GABA and Nav_v1.1 co-immunostained cells from F/F:Dlx-Cre⁻ tissue: FC, frontal cortex; MC, motor cortex; SS, somatosensory cortex; VC, visual cortex; s, superficial layers; d, deep layers; DG, dentate gyrus; CA1, hippocampal CA1; CA3, hippocampal CA3 (*=p<0.05, **=p<0.01, °=p=0.08). (H) Number of excitatory pyramidal neurons immunostained for Nav_v1.1 channels but not GABA in the pyramidal layers of SS and VC. (I) Decreased Nav_v1.1 immunostaining intensity is observed only in F/F:Dlx-Cre⁺ cells also co-expressing GABA (IN) and not in pyramidal cells (Pyr) or dentate granule neurons (DGN) expressing Nav_v1.1 alone (IN, interneurons; Pyr, pyramidal cells; DGN, dentate granule neurons) (Scale bar, 100 μm).



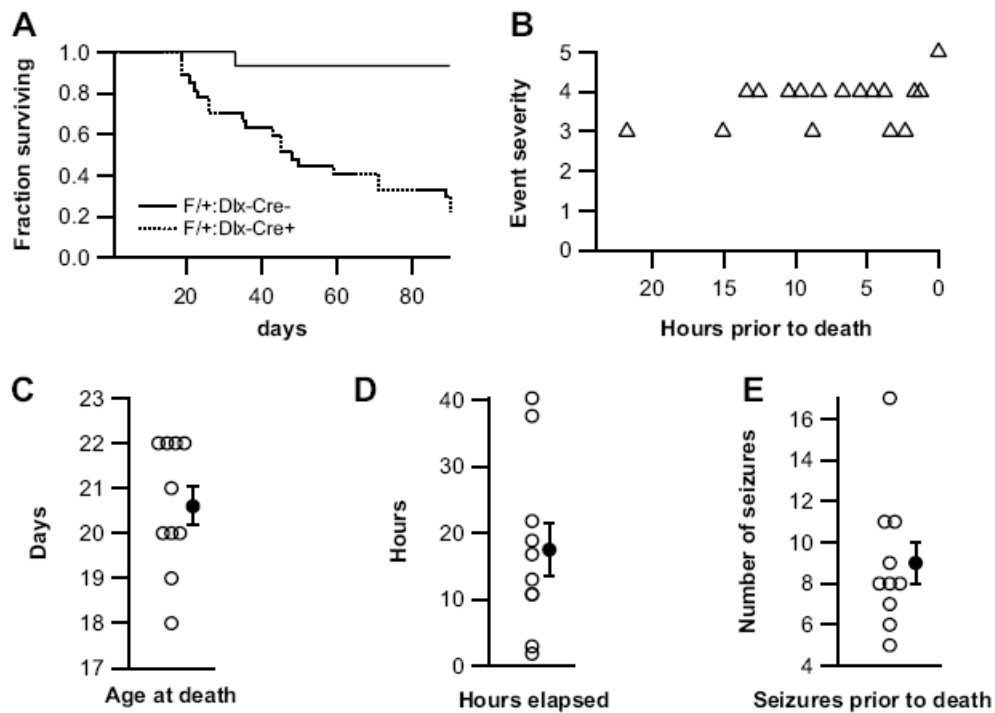


Figure 2.3. Conditional heterozygotes experience excess death and spontaneous seizures. (A) Survival of F/+;Dlx-Cre- and F/+;Dlx-Cre+ mice. The fraction of each genotype surviving is plotted versus postnatal day (F/+;Dlx-Cre- n=45, F/+;Dlx-Cre+ n=27). **(B)** Representative example of spontaneous seizure progression in a F/+;Dlx-Cre+ mouse. The Racine Score of each seizure is plotted as a function of the time before death (t = 0 at time of death). **(C)** Graphical representation of the age of death in animals monitored for spontaneous seizure. Average age of death: 20.6 ± 0.4 days (n=10). **(D)** Graphical representation of time elapsed from initial seizure onset to death in animals monitored for spontaneous seizures. Average latency to death: 17.9 ± 3.9 hours (n=10). **(E)** Graphical representation of the number of seizures preceding death in animals monitored for spontaneous seizures. Average number of seizures prior to death: 9 ± 1 seizure (n=10).

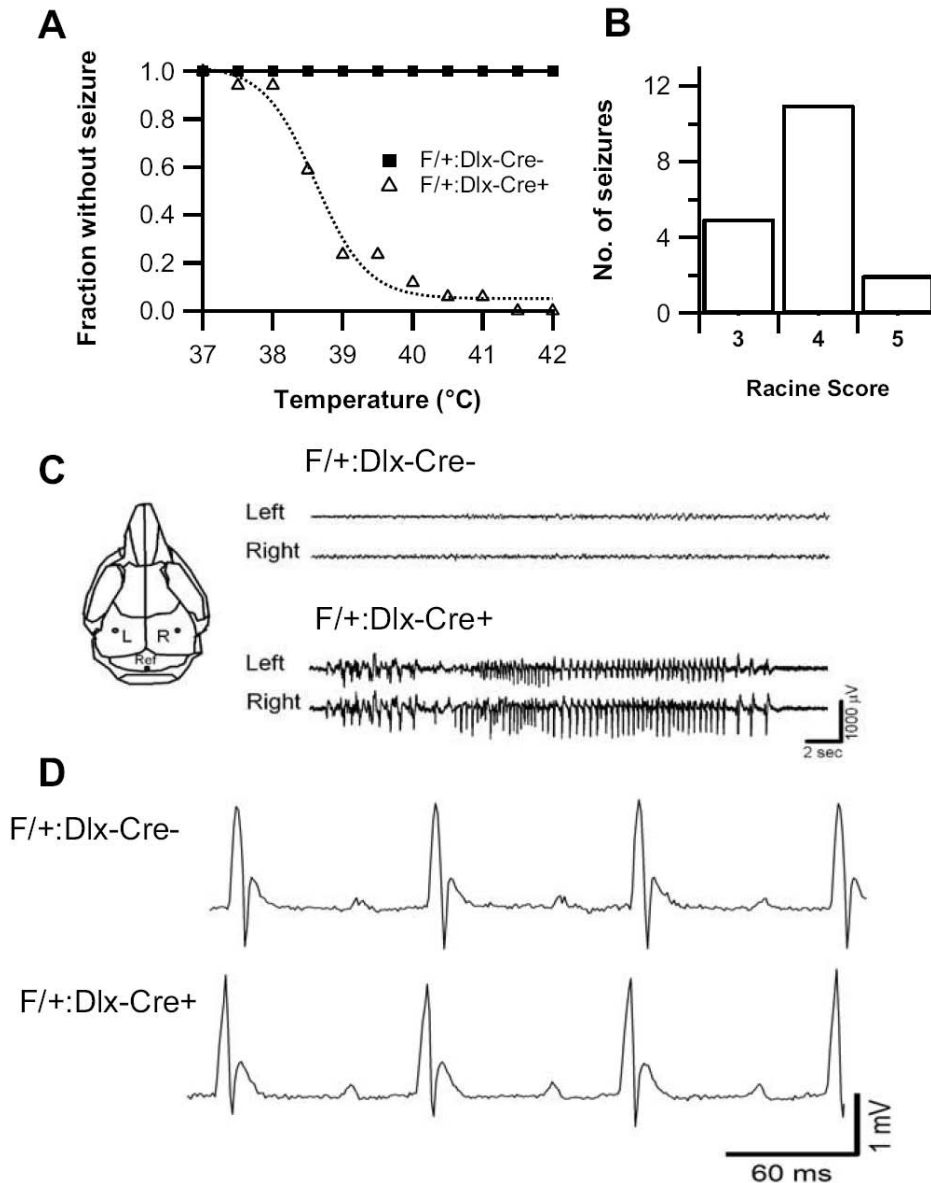


Figure 2.4. Conditional heterozygotes experience evoked behavioral and electrographic seizures (A) Thermally-induced seizures were evoked in all F/+;Dlx-Cre+ animals with a mean temperature of 39°C. No F/+;Dlx-Cre- mice presented with seizure (F/+;Dlx-Cre- n=10, F/+;Dlx-Cre+ n=17). (B) Distribution graph of thermally induced seizure severity in F/+;Dlx-Cre+ mice. (C) Representative EEG traces of F/+;Dlx-Cre- (n=5) and F/+;Dlx-Cre+ (n=4) mice during thermal induction at P24. Top, F/+;Dlx-Cre- mouse at 39.5°C. Bottom, F/+;Dlx-Cre+ mouse during GTC seizure at 39.5°C. (D) Representative ECG traces of F/+;Dlx-Cre- (n=5) and F/+;Dlx-Cre+ (n=6) mice at rest. Top, F/+;Dlx-Cre- mouse: heart rate, 672.2 ± 24 ; PR interval, 32.8 ± 2 ; QRS interval, 9.4 ± 1 ; QT interval, 23.5 ± 5.5 . Bottom, F/+;Dlx-Cre+ mouse: heart rate, 658.4 ± 57 ; PR interval, 32.3 ± 2 ; QRS interval, 8.3 ± 1 ; QT interval, 19.7 ± 1.5 ($p > .05$ for all parameters).

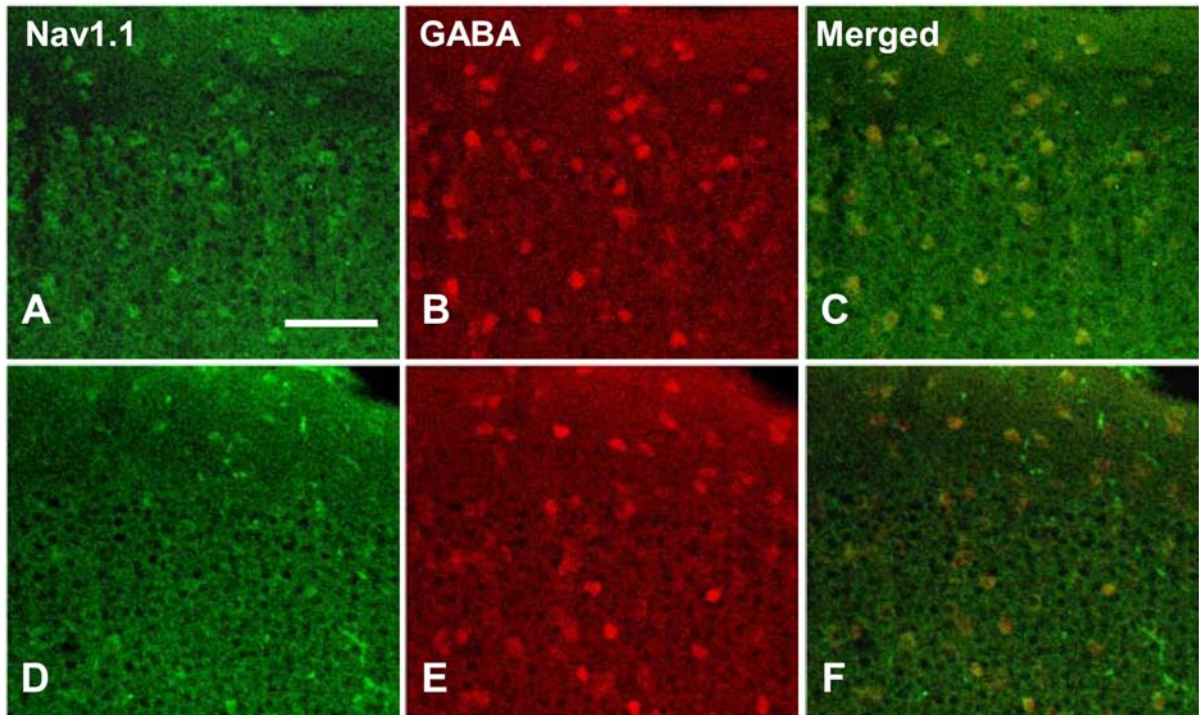


Figure 2.5 Dlx1/2-I12b-Cre preferentially decreases Nav1.1 expression in interneurons. (A-F) Representative immunostaining for Nav1.1 (green), GABA (red) and merged (yellow) in the superficial visual cortex of F/F:Dlx-Cre- (A-C) and F/F:Dlx-Cre+ (D-F) demonstrates a selective loss of Nav1.1 staining only in GABA positive cells in F/F:Dlx-Cre+ sections (D). (Scale bar, 100 μ m)

Chapter 3 - Comorbidities measured in interneuron-specific conditional Nav1.1 mutants

The results on context-dependent fear conditioning in this chapter are presented in a manuscript under review by: Han S, Tai C, Westenbroek RE, Yu FH, Cheah CS, Potter GB, Rubenstein JL, Scheuer T, de la Iglesia HO, Catterall WA. Autism-related behaviors and cognitive deficit in a mouse model of Dravet Syndrome and rescue by enhancing inhibitory neurotransmission.

Introduction

This chapter will focus on comorbidities studied in the animal model characterized in Chapter 2. Dravet Syndrome (DS) is a well characterized condition frequently associated with mutations in *SCN1A* the human gene encoding the voltage-gated sodium channel Na_v1.1. Characteristics of this syndrome include early onset febrile seizures that develop over time to include generalized afebrile seizures, myoclonus, and other seizures types (Dravet, 2011). While frequently considered an epilepsy syndrome, DS also encompasses a larger set of comorbidities in addition to pharmaco-resistant seizures, all of which contribute to a lower quality of life for affected individuals (Brunklau et al., 2011).

Patients with DS develop normally up to the time of their first seizure event, after which DS patients' development and cognitive abilities begin to slow (Guerrini and Falchi, 2011; Ragona, 2011). The long-term outcome for cognitive function is poor and children are often left with severe cognitive impairment (Wolff et al., 2006; Genton et al., 2011). Studies have been inconclusive regarding the relationship between seizure severity and cognitive

impairment with some finding a positive association between seizure control and better cognitive outcomes, and others showing no correlation whatsoever (Guerrini and Falchi, 2011).

In addition to seizures and permanent cognitive impairment, individuals are also afflicted by a variety of other co-morbidities including ataxia, sleep disturbance, learning deficits, and a higher rate premature death than is seen in most other epilepsy syndromes (Brunklaus et al., 2011; Sakauchi et al., 2011). Patients with DS experience a progressive decline in coordination beginning after 4 years of age. By 13 years of years of age, the vast majority of patients have developed a crouching posture and a distinctive skeletal misalignment (Rodda et al., 2012). In adults, patients have been reported to have bradykinesia and difficulty with coordination (Martin et al., 2010b). Studies in the global knockout animals have revealed a modest deficiency in foot placement coordination as well as circadian dysfunction (Kalume et al., 2007; Han et al., 2012).

Sodium channel mutations can generate seizures and the associated comorbidities of DS through either cell death or network dysfunction. Studies of cognitive impairment and movement disorders have failed to find a correlation between severity of the impairment and regional atrophy or damage by MRI (Martin et al., 2010b; Guerrini and Falchi, 2011). These studies suggest loss of function of Na_v1.1 channels as the cause of not just the epilepsy but additional comorbidities seen in DS. Utilizing our interneuron specific conditional heterozygous animals (F/+;Dlx-Cre+), we have investigated the role of loss of

Na_v1.1 in inhibitory neurons of the forebrain in motor coordination and cognitive performance.

Results

Mutant animals on CD1:C57BL/6 background are not ataxic

Previous ataxia studies in our lab used animals heterozygous for a global deletion of Na_v1.1 (global HET) that has been extensively characterized by our research group (Yu et al., 2006). In that study global HET animals demonstrated a subtle ataxia, as they were not precise and consistent in their placements of the forepaw and hind paw during locomotion, while other metrics measured remained unchanged when compared to wildtype (WT) littermate controls (Kalume et al., 2007). A study of motor coordination in adults with DS did not find evidence of cerebellar atrophy and the authors suggest the nature of coordination deficit may be of frontal lobe origin (Martin et al., 2010b). To investigate the potential role of loss of Na_v1.1 specifically in forebrain interneurons we analyzed the gait of our F/+;Dlx-Cre+ animals previously described in Chapter 2.

Gait analysis of F/+;Dlx-Cre+ and their F/+;Dlx-Cre- littermate controls were determined by a modified footprint test, and stride length, width and forepaw/hindpaw placement were measured as previously described (Kalume et al., 2007). Measurements of hindpaw and forepaw positions showed no difference in the stride length between genotypes (Figure 3.1A; F/+;Dlx-Cre+, 5.44±.08 cm; F/+;Dlx-Cre-, 5.52±.18). The most notable deficit in global

HET animals was in their precision of hindpaw and forepaw placement. However, when F/+;Dlx-Cre⁺ and F/+;Dlx-Cre⁻ mice were analyzed for the precision of forepaw/hindpaw placement, animals of both genotypes performed equally well, indicating that their performance is not impaired by loss of Na_v1.1 in forebrain interneurons (Figure 3.1B; F/+;Dlx-Cre⁺, .21±.03 cm; F/+;Dlx-Cre⁻, .25±.03 cm).

We have previously seen a strong genetic background effect on survival in the global HET animals. Our conditional mutants were bred on a mixed CD1:C57BL/6 background instead of pure C57BL/6, as in our previously studied global HET animals. It is possible that the differences in forepaw/hindpaw placement between global HET and conditional mutant animals were the result of genetic background effects. To address this concern, we repeated the gait analysis in global HET animals on the mixed CD1:C57BL/6 background. CD1:C57BL/6 animals were generated by crossing a CD1 global HET male with a WT C57BL/6 female. The resulting animals, F1 progeny, were global HETs and on the same CD1:C57BL/6 background as our conditional mutants. As in our CD1:C57BL/6 conditional mutants, no difference in stride length (Figure 3.1A; HET, 5.25±.12 cm; WT, 5.21±.15 cm) or precision of hindpaw/forepaw placement (Figure 3.1B; HET, .28±.04 cm; WT, .36±.03 cm) were found in CD1:C57BL/6 global HETs. Additionally, no statistically significant differences were found when data across all 4 genotypes were taken into consideration. Taken together, these data demonstrate that both conditional loss of Na_v1.1 channels in forebrain interneurons and global loss of Na_v1.1 have no effect on ataxia in animals on a mixed CD1:C57BL/6 background.

Specifically deleted *Scn1a* Mutant animals on CD1:C57BL/6 background do not exhibit spasticity

In the original ataxia study conducted by Kalume, et al., C57BL/6 global HET animals were also tested for spasticity. To determine if interneuron specific deletion of $Na_v1.1$ has an effect on spasticity, F/+;Dlx-Cre+ and F/+;Dlx-Cre- littermate controls were tested for motor deficits in the tail suspension paradigm. There was no statistically significant difference in the reaching time between F/+;Dlx-Cre+ and F/+;Dlx-Cre- animals. (Figure 3.2; F/+;Dlx-Cre+, $90 \pm 3.6\%$; F/+;Dlx-Cre-, $82 \pm 1.8\%$) To examine the genetic background effects, these experiments were repeated on CD1:C57BL/6 global HET animals. Similarly, no differences were seen between WT and global HET animals. (Figure 3.2; HET, $93 \pm 1.6\%$; WT, $84 \pm 3.9\%$) No statistically significant differences were seen when data was compared across all 4 genotypes. As in our ataxia studies, we found both global loss of $Na_v1.1$ and conditional loss of $Na_v1.1$ channels in forebrain interneurons have no effect on spasticity in animals on a mixed CD1:C57BL/6 background.

F/+;Dlx-Cre+ animals have impaired cognitive abilities

Children with DS have been shown to have impaired learning abilities and reduced overall cognitive function (Wolff et al., 2006). Using context-dependent fear conditioning the role of specific loss of $Na_v1.1$ in interneurons in spatial learning and memory was studied. Both F/+;Dlx-Cre+ and F/+;Dlx-Cre- animals actively explore the test chamber when

first introduced and mice of both genotypes demonstrate similar freezing behavior immediately following a mild foot shock. However, when F/+;Dlx-Cre+ animals were returned to the test chamber they display significantly less freezing behavior than their F/+;Dlx-Cre- littermates in short-term (30 min) and long-term (24 hour) tests (Figure 3.3). These data suggest that F/+;Dlx-Cre+ animals experience fear but either do not associate that fear with a specific environment or fail to remember that association because these mice have normal performance in the novel object recognition test, they do not have a general deficit in memory and may therefore be unable to encode spatial context. (Han, et al., 2012b). Additionally, our global heterozygous *Scn1a* mutant DS mouse model also displays neuropsychiatric dysfunctions, such as impaired social interaction, hyperactivity, and anxiety-like behaviors suggesting a larger role for loss of Na_v1.1 in interneurons beyond control of overall excitability (Han et al., 2012b).

Discussion

In addition to seizures, individuals with DS also have cognitive and behavioral impairments, sleep disturbances, impaired visual function and a higher rate of death than other seizure disorders (Wolff et al., 2006; Chieffo et al., 2011a; Genton et al., 2011; Guerrini and Falchi, 2011). All of these comorbidities contribute to decreased quality of life for individuals with this condition (Brunklau et al., 2011). Studies in our animal model of DS have revealed deficiencies in ataxia and circadian rhythm (Kalume et al., 2007; Han et al., 2012).

Using our F/+:Dlx-Cre+ animals we sought to determine the specific role of Na_v1.1 channel loss in inhibitory interneurons on ataxia and cognitive function. Our results demonstrate that loss of Na_v1.1 in forebrain interneurons of mice on a mixed CD1:C57BL/6 background has no effect on motor coordination in the administered tests. Furthermore, we determined that global heterozygous loss of Na_v1.1 in animals on a CD1:C57BL/6 background at the same age also had no effect on motor coordination. This stands in contrast to what was seen in animals on a pure C57BL/6 strain at the same age. The ataxia in C57BL/6 global HET animals was restricted to the precision of forepaw and hindpaw placement and this deficit was absent in both our F/+:Dlx-Cre+ and global HET animals on a mixed CD1:C57BL/6 background, suggesting that there may be an additional genetic background effect with regard to ataxia.

The age at which ataxia is measured may be an avenue of research to pursue in the future. Recent work has shown that individuals with DS have a progressive deterioration of gait that begins to be most noticeable during adolescence, suggesting that in both the global and conditional deletion mutants an older timepoint should be considered in future studies (Rodda et al., 2012).

Studies of cognitive abilities in F/+:Dlx-Cre+ animals demonstrate that loss of Na_v1.1 in interneurons is sufficient to impair context-dependent fear conditioning, suggesting that deficiencies in cognitive performance in both humans and animal models is due to reduced Na_v1.1 activity in forebrain interneurons. Additionally, other studies performed in our lab

with these mice have shown deficits in neuropsychiatric functions, such as impaired social interaction, hyperactivity, anxiety-like behavior (Han et al., 2012b).

The F/+;Dlx-Cre+ animals have shown the impact of interneuron specific loss of Na_v1.1 function on seizure generation (Chapter 2) and cognitive function. Moving forward this mouse model will continue to be an important and effective tool in the lab as we tease apart the contribution of interneuron specific Na_v1.1 channels to premature death and other comorbidities of DS.

Methods

Animal care

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Washington. Na_v1.1^{+/-} animals were maintained independently on both C57Bl/6 and CD1 backgrounds as described previously (Yu et al., 2006). Interneuron specific conditional deletion of Na_v1.1 was achieved by crossing F/F female mice maintained on a C57Bl/6 background with Dlx-1/2-i12b-Cre animals (Dlx-Cre;Potter et al., 2009) maintained on a CD1 background as described in Chapter 2. The F1 progeny of this cross were used to maintain a consistent genetic background and all experimental comparisons were between littermates.

Gait analysis

For gait analysis, we used a modified footprinting test. F/+;Dlx-Cre⁺ and F/+;Dlx-Cre⁻ animals walked through a covered 40cm opaque Plexiglas tunnel across a clear Plexiglas platform suspended ~40cm above the benchtop while being videotaped from below. Individual footprints from complete runs are marked when reviewing video frame by frame (Adobe Flash) and exported as a still image used to measure stride length, width and precision of ipsilateral forepaw and hindpaw placement (Kalume et al., 2007).

Measurement of Spasticity

Skeletal muscle spasticity was tested by hanging tail suspension. F/+;Dlx-Cre⁺ and F/+;Dlx-Cre⁻ animals are suspended by their tail ~1 cm above a wire cage top and allowed to reach for the cage for 20 seconds. The time the animals spent intentionally reaching for the bars is scored from video review and represented as a percentage of the total time spent reaching (Kalume et al., 2007).

Contextual fear conditioning

The contextual fear conditioning chamber is a square arena (25 x 25 cm) with clear plexiglass walls and the metal grid floor that consists of a circuit board that delivers shocks to metal grids (Coulborun Instruments). An analog camera, as a part of a whole fear

conditioning system is attached on the top of the chamber. The camera and the circuit board are connected to the personal computer and the software (Freeze Frame 2.0, Actimetrics) controls the circuit and records the data. The chamber was cleaned by 5% acetic acid and swiped with paper towels between each session. In the habituation session, a test mouse was placed in the chamber and allowed to explore the chamber for 2 min. Immediately after the habituation, the test mouse received single mild foot shock (2 sec, 0.7 mA). After staying in the chamber one more minute, the mouse was removed from the chamber. For the 30-min short-term memory test, the mouse was returned to the context 30 min after the end of the training session. For 24 hr long-term memory test, the mouse was returned to the context 24 hr after the training session. The movement of mice was recorded by a USB webcam (LifeCam HD-6000, Microsoft) and PC-based video capture software (WinAVI Video Capture, ZJMedia Digital Technology) for 2 min. Recorded video file was further analyzed by off-line video tracking software (EthoVision XT 7.0, Noldus Technology). Average freezing time in 1 min was measured in each session. All data shown are means \pm s.e.m. and analyzed using Student's two-tailed, unpaired t-test.

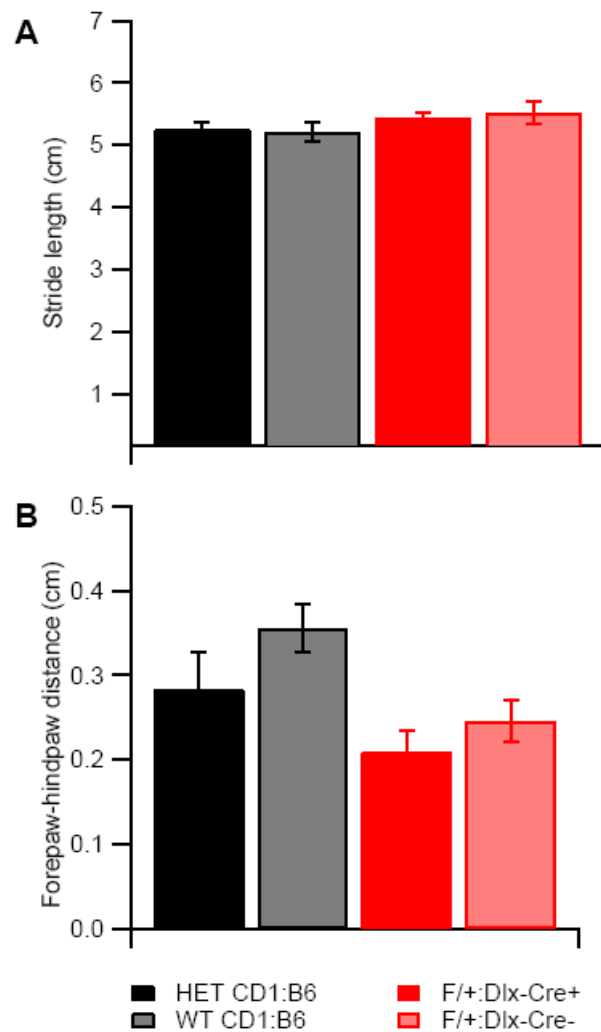


Figure 3.1. Footprint analysis of global HET, WT, F/+Dlx-Cre+, and F/+Dlx-Cre- animals on CD1:C57Bl/6 background shows no difference. (A) Stride length measurements reveal no significant difference between global HET, WT, Dlx+:F/+, and Dlx-:F/+ animals (global HET, 5.25±.12 cm; WT, 5.21±.15 cm; F/+Dlx-Cre+, 5.44±.08 cm; F/+Dlx-Cre-, 5.52±.18). (B) Center to Center measurements of forepaw and hindpaw placement revealed no significant difference between the four genotypes in the precision of paw placement (HET, .28±.04 cm; WT, .36±.03 cm; F/+Dlx-Cre+, .21±.03 cm; F/+Dlx-Cre-, .25±.03 cm). HET, n=17; WT, n=9; F/+Dlx-Cre+, n=10; F/+Dlx-Cre-, n=8

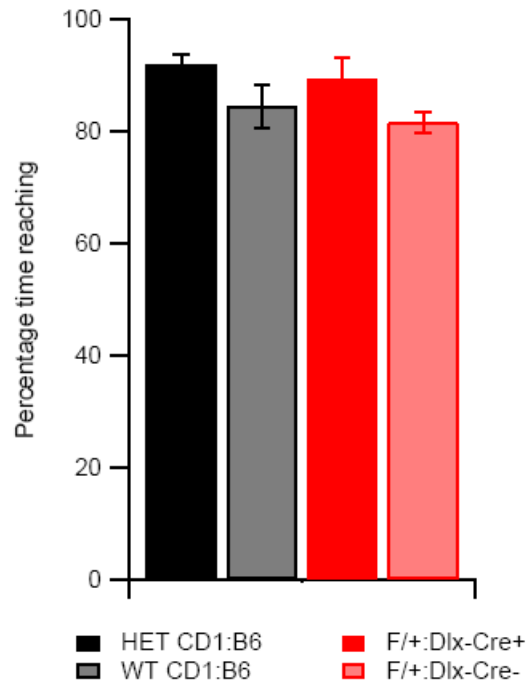


Figure 3.2. No difference in spasticity between HET, WT, F/+;Dlx-Cre+, and F/+;Dlx-Cre- animals on CD1:C57Bl/6 background. Utilizing hanging tail suspension as a measure of total body spasticity, no statistically significant differences in time spent reaching were seen between all four genotypes (HET, 93±1.6%; WT, 84±3.9%; F/+;Dlx-Cre+, 90±3.6%; F/+;Dlx-Cre-, 82±1.8%). HET, n=16; WT, n=9; F/+;Dlx-Cre+, n=10; F/+;Dlx-Cre-, n=8

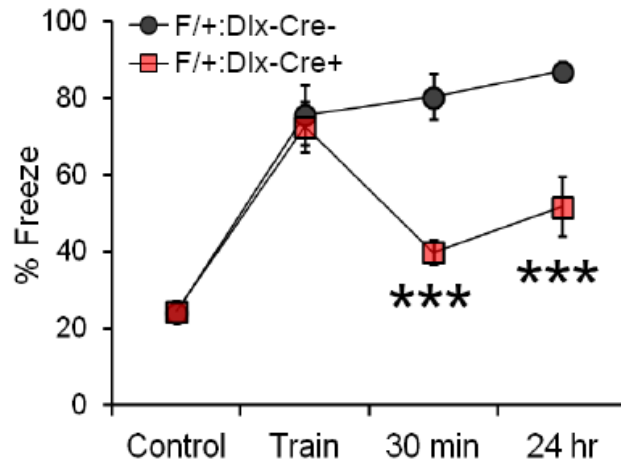


Figure 3.3. Fear conditioning on F/+;Dlx-Cre+ and F/+;Dlx-Cre- mice demonstrates impaired memory. In the contextual fear conditioning test, F/+;Dlx-Cre+ mice display profound deficit in short-term (30 min) and long-term (24 hr) memory of 2 s mild foot shock (0.5 mA)-associated context, but normal fear response immediately after the training (Train) when compared to F/+;Dlx-Cre- mice. All data shown are means \pm s.e.m. from 7 – 9 mice per genotype. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (Han et al., 2012b)

Chapter 4 - Sodium channel Na_v1.3 as a potential precipitating factor and genetic modifier in Dravet Syndrome

This chapter is presently in preparation as a manuscript for publication with the authors: Christine S Cheah, Ruth E Westenbroek, William H Roden, John C Oakley, Laura A Jansen, John Wood, William A Catterall

Abstract

The voltage-gated sodium channels Na_v1.1, Na_v1.2, Na_v1.3, and Na_v1.6 are dynamically expressed in the developing central nervous system and are essential for proper network function. Loss-of-function mutations in *SCN1A*, the gene encoding Na_v1.1, leads to Dravet Syndrome (DS), a devastating genetic epilepsy. We tested whether changes in expression of Na_v1.3 may be a precipitating factor or genetic modifier in DS. We found that Na_v1.3 is expressed at high levels in embryonic life in mice and humans, and its expression declines after birth coincident with increasing Na_v1.1 channel expression. The onset of seizures in mice and humans follows the developmental decrease in Na_v1.3 to less than half of its maximal expression level suggesting that its loss, coupled with failure of normal expression of Na_v1.1 channels, may contribute to the time of seizure onset in DS. We tested whether genetic deletion of Na_v1.3 channels would exacerbate the early phase of DS in mice and found that heterozygous loss of Na_v1.3 does not lead to impaired survival or increased sensitivity to thermally induced seizures, consistent with the conclusion that loss of more

than half of Na_v1.3 channels is necessary for onset of DS. Together, our results provide support for the hypothesis that declining expression of Na_v1.3 channels below 50% of maximum, in the face of heterozygous loss-of-function mutation of the Na_v1.1 channel, may be one of the precipitating factors contributing to the time of onset of DS.

Introduction

Voltage-gated sodium channels (Na_v) are expressed throughout the central and peripheral nervous systems. Each sodium channel protein is composed of a 260 kDa pore forming alpha subunit and one or more auxiliary beta subunits (Catterall, 2000). The Na_v1.1, Na_v1.2, Na_v1.3, and Na_v1.6 isoforms are expressed in brain. These proteins are responsible for initiation and propagation of action potentials, which are required for normal brain function, and regulation of their expression, subcellular localization, and functional activity allows fine control of network excitability (Catterall, 2000). Na_v1.1 and Na_v1.3 are found primarily in the cell soma and proximal dendrites, while Na_v1.2 is localized primarily in unmyelinated axons. Na_v1.6 controls action potential initiation and propagation in myelinated axons and is also expressed in cell bodies and dendrites of some neurons (Westenbroek et al., 1989, 1992; Vacher et al., 2008). This channel distribution is similar in human and rodent brains (Vacher et al., 2008).

Due to their distinct subcellular localization, dysfunction of any isoform of these channels can lead to epilepsy, chronic pain, periodic paralysis, or cardiac arrhythmia (Lehmann-Horn and Jurkat-Rott, 1999; Waxman et al., 2000). Loss of function of Na_v1.1 leads to a distinct

epilepsy phenotype known as Dravet Syndrome (DS) or Severe Myoclonic Epilepsy of Infancy. DS is a progressive neurological condition that begins with febrile seizures in infancy and progresses with development to include drug-refractory spontaneous seizures, cognitive impairment, mild ataxia, and premature death (Dravet, 2011).

The expression of all sodium channels is developmentally regulated. In rat, Na_v1.2 and Na_v1.6 expression increases continually from birth, but Na_v1.1 and Na_v1.3 expression patterns are inversely related to each other. Na_v1.1 expression is low at birth and increases to a steady state level by the third week of life, while Na_v1.3 expression levels are highest just prior to birth and decline in postnatal life to a low level by four weeks (Gordon et al., 1987; Beckh et al., 1989; Felts et al., 1997; Gong et al., 1999). In humans, the levels of Na_v1.3 protein observed in immunocytochemical studies remain higher in some brain regions in adulthood compared to rats (Whitaker et al., 2001), but the developmental time course of expression of these channels is not known.

Our lab has developed a mouse model of DS by globally deleting exon-26, the last protein-coding exon of *Scn1a*, the gene that encodes Na_v1.1 protein (Yu et al., 2006). This mouse model recapitulates many of the properties of the human disease, including progressively increasing severity of seizures, co-morbidities, and premature death (Kalume et al., 2007; Oakley et al., 2009; Han et al., 2012). A second mouse model of DS, created by targeted gene mutation, has similar properties (Ogiwara et al., 2007). Mice heterozygous for the gene deletion (*Scn1a*^{+/-}) develop sensitivity to thermally induced and spontaneous seizures and often experience premature death in the fourth week of life (Yu et al., 2006; Oakley et al., 2009).

Inherited epilepsies are susceptible to genetic background effects, and individual DS patients with complete loss-of-function mutations have symptoms of varying severity (Singh et al., 2001; Guerrini et al., 2010; Suls et al., 2010). Similarly, the premature death observed in our *Scn1a*^{+/-} DS animals has a strong dependence on genetic background. Animals bred on a pure C57Bl/6 background were much more susceptible to death than those bred on a pure 129SvJ background, and animals bred on a mixed F1, C57Bl/6:129SvJ background died at a rate intermediate to the two pure strains (Yu et al., 2006). Na_v1.6 has been shown to be a genetic modifier whose heterozygous deletion abolishes premature death in our animal model of DS (Martin et al., 2007), and immunohistochemical studies on our *Scn1a*^{-/-} animals showed a compensatory increase in Na_v1.3 in the hippocampus as Na_v1.1 protein levels decline (Yu et al., 2006), raising the possibility of genetic compensation by Na_v1.3 channels.

In this study we have defined the time course of expression of Na_v1.1 and Na_v1.3 channels in mouse and human cerebral cortex to search for a temporal correlation with onset of DS symptoms, and we have tested the possibility that Na_v1.3 may be a genetic modifier of DS in mice using a *Scn3a* knockout mouse line.

Results

Expression of Na_v1.1 and Na_v1.3 channels in mouse and human brain

Previous studies of rat brain showed that Na_v1.3 mRNA and protein is expressed at high levels in embryonic life and expression declines in the first three weeks after birth, whereas

Na_v1.1 channel expression is first observed in early postnatal development and reaches its maximum expression level by four weeks of age (Beckh et al., 1989; Felts et al., 1997). Immunoblotting on membrane proteins isolated from wildtype C57Bl/6 mouse cortex ranging in age from postnatal day 1 (P1) through P30 with antibodies specific for Na_v1.1 and Na_v1.3 showed an expression pattern similar to that seen in rat (Figure 4.1A). However, the decline in Na_v1.3 channel protein was more rapid, reaching its lowest levels at P14, and the rise in Na_v1.1 channels was later, peaking at P30, than in rat.

In order to compare channel expression patterns in human brain during development to that observed in rodents, membrane proteins were isolated from non-disease associated human postmortem cerebral cortex tissue between 1.5 and 30 months. Immunoblotting with antibodies specific for Na_v1.1 and Na_v1.3 demonstrated Na_v1.1 protein expression parallels rodent expression and is lowest at birth and steadily increases to its highest level by 20 months (Figure 4.1B). Na_v1.3 protein expression also parallels rodent expression patterns and is detected at its highest levels at birth and decreases to its lowest level at 6 months of age (Figure 4.1B). The time at which the decline of Na_v1.3 protein levels and the rise of Na_v1.1 expression cross (approximately 5-6 months) correlates closely with seizure onset in patients with DS (approximately 6 months; Table 4.1).

Patients with DS are diagnosed using behavioral and electrographic symptoms, and it is not essential to have an identified *SCN1A* mutation to obtain a DS diagnosis (Petrelli et al., 2011). Children diagnosed with DS develop normally until the time of seizure onset, which occurs between 5 and 11 months, with an average age of onset of 5.7 months (Table 4.1;

Dravet, 2011). After initial seizure onset, DS progresses rapidly to multiple forms of spontaneous seizures, including generalized tonic-clonic seizures, resistance to drug therapy, developmental delay, and permanent mental and physical deficiencies (Ragona et al., 2010, 2011; Chieffo et al., 2011b; Lim et al., 2011; Petrelli et al., 2011).

Na_v1.3 as a potential genetic modifier in DS

Although most DS mutations truncate the Na_v1.1 channel protein and are expected to cause complete loss of function, there is a striking variability of disease penetrance and seizure severity among individual patients, indicating a strong genetic background effect on disease progression (Singh et al., 2001; Guerrini et al., 2010; Suls et al., 2010). Similarly, our DS mice have variable survival rates in different mouse strains with identical gene mutations, indicating a strong genetic background effect with regard to survival. In our previous studies, survival of *Scn1a*^{+/-} animals on a pure C57Bl/6 background was less than 30% of that observed for progeny on a pure 129SvJ background heterozygous for the identical mutation (Yu et al., 2006). The genes that contribute to this genetic background effect are unknown, but Na_v1.3 immunostaining in GABAergic interneurons of the hippocampus increases with loss of Na_v1.1 channels in DS mice, suggesting *Scn3a*, the gene encoding Na_v1.3, as a potential genetic modifier (Yu et al., 2006). These data, taken together with the somatic location and complementary temporal development of Na_v1.1 and Na_v1.3 in humans and rodents, suggest Na_v1.3 as a potential positive genetic modifier of disease severity and survival.

Animals haploinsufficient for Na_v1.3 (*Scn3a*^{+/-}) were indistinguishable from WT littermates and did not have any obvious neurological deficits (Nassar et al., 2006). If Na_v1.3 is influencing the survival of *Scn1a*^{+/-} animals, we would have expected animals heterozygous for gene deletions in both *Scn1a* and *Scn3a* (*Scn1a*^{+/-}:*Scn3a*^{+/-} double Het) to have died earlier and in greater numbers than their *Scn1a*^{+/-} littermates. To directly test this hypothesis C57Bl/6 *Scn1a*^{+/-} mice were crossed with C57Bl/6 *Scn3a*^{+/-} mice. We found that survival was not diminished in the *Scn1a*^{+/-}:*Scn3a*^{+/-} double Hets compared with the *Scn1a*^{+/-} population at any time (Figure 4.2A; p>0.05). Both *Scn1a*^{+/-} and *Scn1a*^{+/-}:*Scn3a*^{+/-} double Het populations experienced frequent premature deaths in the fourth week of life, and at 60 days only approximately 60% of the mice of all genotypes remained viable (Figure 4.2A). More additional deaths occurred in the *Scn1a*^{+/-} population after 60 days, such that only 25% of *Scn1a*^{+/-} survived at 90 days compared to 46% of *Scn1a*^{+/-}:*Scn3a*^{+/-} double Het animals. It was surprising that *Scn3a*^{+/-} animals on the C57Bl/6 genetic background demonstrated greater premature death than Na_v1.3 single mutants, with death beginning at P31 and only 66% of animals surviving after 90 days. As expected, no WT animals died over the course of our observations (Figure 4.2A).

Given the evident sensitivity to genetic background and the intermediate rate of death previously seen in animals on a mixed background, male 129SvJ *Scn1a*^{+/-} were crossed with female C57Bl/6 *Scn3a*^{+/-} mice to generate *Scn1a*^{+/-}:*Scn3a*^{+/-} double Hets on a background similar to that used in our original studies (Yu et al., 2006). In *Scn1a*^{+/-} animals a dramatic increase in death begins in the fourth week of life with 30% of the population remaining

after 90 days. Survival of *Scn1a*^{+/-}:*Scn3a*^{+/-} double Hets was not more impaired than *Scn1a*^{+/-} animals in the 50:50 129SvJ:C57Bl/6 genetic background at P90, with 40% of the population remaining (Figure 4.2B; p>0.05). However, the rate of death in *Scn1a*^{+/-}:*Scn3a*^{+/-} double Hets was slower and at P50 significantly more *Scn1a*^{+/-}:*Scn3a*^{+/-} double Het animals remain alive than *Scn1a*^{+/-} littermates (Figure 4.2B; p<0.05). WT and *Scn3a*^{+/-} populations showed little to no death during the same period, with a single death observed in the WT population on P22 and no death seen in *Scn3a*^{+/-} population. It was surprising to observe the improvement in survival of *Scn1a*^{+/-}:*Scn3a*^{+/-} double Hets compared to *Scn1a*^{+/-} mice in the 50:50 129SvJ:C57Bl/6 background. One possible mechanism underlying this effect could be reduced Na_v1.3 expression in excitatory neurons, which also express this channel in embryonic and early postnatal life.

Na_v1.3 channels and susceptibility to thermally induced seizures

Patients with DS (Dravet, 2011) and our mouse model of this disease (Oakley et al., 2009) are susceptible to seizures evoked by elevation of body temperature. 129SvJ *Scn1a*^{+/-} animals were crossed with C57Bl/6 *Scn3a*^{+/-} animals and the resulting F1 progeny were analyzed in our thermal induction protocol at P21. When the core body temperature of 129SvJ:C57Bl/6 *Scn1a*^{+/-} animals was elevated, seizures were induced in all animals, with a mean core body temperature of 38.7°C (Figure. 4.3; *Scn1a*^{+/-}, n=6). When the core body temperature of *Scn1a*^{+/-}:*Scn3a*^{+/-} double Het littermates was elevated, seizures were induced in all animals, with a mean seizure onset temperature of 38.1°C (Figure 4.3; *Scn1a*^{+/-}

:*Scn3a*^{+/-} double Het, n=6). All *Scn1a*^{+/-} mice had seizures at P21, whether or not the Na_v1.3 mutation was also present. As expected, no WT or *Scn3a*^{+/-} animals had seizures (WT, n=14; *Scn3a*^{+/-}, n=12; Figure 4.3).

Discussion

Loss of Na_v1.3 channels precedes seizure onset in mouse and human

The results of these experiments demonstrate that the decline in Na_v1.3 and the rise of Na_v1.1 expression in human cerebral cortex occurs in the same time frame as the initial presentation of seizures in patients diagnosed with DS, both at 6 months. Similarly, the timing of the decline of Na_v1.3 and the rise of Na_v1.1 expression in rodents precedes the third week of life (Figure 4.1A; Beckh et al., 1989), and our mouse model of DS is not susceptible to thermally induced or spontaneous seizures before P21 (Oakley et al., 2009). These data in human and mouse suggest that the timing of DS onset is a result of the failure of Na_v1.1 to fully replace the loss of Na_v1.3. Thus, loss of Na_v1.3 channels may be one of the precipitating factors that initiates the disease process in DS.

Haploinsufficiency of Na_v1.3 channels does not exacerbate DS

The similar somatic localization of Na_v1.1 and Na_v1.3 and their complementary temporal expression patterns make Na_v1.3 an attractive channel to compensate for loss of Na_v1.1

through up-regulation. However, survival of *Scn1a*^{+/-}:*Scn3a*^{+/-} double Hets is no more impaired than in *Scn1a*^{+/-} alone in both pure C57Bl/6 and mixed 129SvJ:C57Bl/6 backgrounds. These results show that loss of one allele of *Scn3a* has no detrimental impact on survival in DS mice.

The lack of effect of haploinsufficiency of Na_v1.3 channels *per se* on survival of DS mice is in agreement with the timing of the loss of Na_v1.3 channels and the onset of seizures in mice and humans. In mice, 50% of Na_v1.3 channels are lost in the first week of life, and nearly all Na_v1.3 channels are lost by P21 when the first susceptibility to seizures is observed in DS mice. If 50% or more of Na_v1.3 channels remained at P21, they might be sufficient to slow or prevent the onset of seizures in DS mice. Similarly in humans, the number of Na_v1.3 channels is reduced to 50% before approximately five months of age but the first signs of DS occur one month later. Thus, in both humans and mice, loss of more than 50% of Na_v1.3 channels in cerebral cortex has occurred before the onset of DS symptoms, consistent with our finding that genetic inactivation of one allele of *Scn3a* does not increase premature death in DS mice. Of course, it is important to note that the time course of loss of Na_v1.3 channels might be different in other brain regions. Nevertheless, our results with genetic crosses of Na_v1.3 and Na_v1.1 deficient mice indicate that haploinsufficiency of Na_v1.3 channel expression globally is not sufficient to increase premature death in DS mice.

In light of the lack of effect of haploinsufficiency of Na_v1.3 channels in DS mice, it would be of great interest to analyze DS mice in which both alleles of *Scn3a* are inactivated.

Unfortunately, the *Scn1a* and *Scn3a* genes are located nearby on chromosome 2 in mouse, and the corresponding *SCN1A* and *SCN3A* genes are localized close together on chromosome 2 in human. Because of this close chromosome location, all chromosomes in our mice that have a deletion in *Scn1a* will have WT *Scn3a* because these genes can differentially segregate only in the extremely unlikely event of a meiotic crossover between them. Thus, the close co-localization of these two genes on chromosome 2 restricts analysis of their effects to double Hets because generation of the *Scn1a*^{+/-}:*Scn3a*^{-/-} Het:KO genotype would require more breeding than is possible in our colony. Therefore, our conclusions concerning the genetic background effects of Na_v1.3 channels are necessarily limited to effects observed in *Scn3a*^{+/-} animals.

Haploinsufficiency of Na_v1.3 channels does not enhance susceptibility to febrile seizures

Our results with *Scn1a*^{+/-}:*Scn3a*^{+/-} double Hets show that haploinsufficiency of Na_v1.3 channels does not increase susceptibility to thermally induced seizures. Animals heterozygous for Na_v1.1 are equally susceptible to thermally induced seizures regardless of whether they have one or both functional alleles of *Scn3a*. These results are also consistent with our results on Na_v1.3 expression in human cerebral cortex, as the onset of febrile seizures is observed after the level of Na_v1.3 channels has declined below 50% of its maximum expression level.

Conclusions

Our results from two different experimental approaches shed new light on the possibility that the Na_v1.3 channel may serve as a precipitating factor or genetic modifier in DS, as suggested by our previous observation that Na_v1.3 channels are up-regulated in the hippocampus of *Scn1a*^{-/-} mice (Yu et al., 2006). First, our studies of the time course of expression of Na_v1.1 and Na_v1.3 channels in human cerebral cortex are consistent with the hypothesis that the loss of more than 50% of Na_v1.3 channels, coupled with failure of normal expression of Na_v1.1 channels, contributes to the onset of symptoms in DS. Second, our studies of *Scn1a*^{+/-}:*Scn3a*^{+/-} double Het mice show that haploinsufficiency of Na_v1.3 channels is not sufficient to exacerbate the premature death or sensitivity to thermally induced seizures in DS mice. Considered together, an important implication of these results is that up-regulation of expression of Na_v1.3 channels to levels approaching 50% of their maximum could potentially provide therapeutic benefit in DS.

Methods

Immunoblotting

Normal postmortem human brain samples were obtained from the NICHD Brain and Tissue Bank for Developmental Disorders at the University of Maryland. Ages analyzed were 1.4, 1.8, 5.2, 5.6, 6, 6.8, 10.7, 20.8, 30.3 months. Immunoblotting of human tissue was done at Seattle Children's Research Institute as previously described in detail (Jansen et al.,

2010). Briefly, membrane proteins were isolated from 100-200 μg of cortical tissue cleaved from the underlying white matter, transferred to PVDF membranes and probed for $\text{Na}_v1.1$ (1/200, rabbit anti- $\text{Na}_v1.1$, Alomone labs), $\text{Na}_v1.3$ (1/200, rabbit anti- $\text{Na}_v1.3$, Alomone labs) and β -tubulin (1/10,000, mouse anti-tubulin, Novus Biologicals). Infrared fluorescence was used for signal detection and quantitation (rabbit anti-Alexa Fluor 680, Molecular Probes; IRDye 800 goat anti-mouse IgG, LI-COR; Odyssey Infrared Imagins System, LO-COR Biosciences). Background was subtracted, and levels of $\text{Na}_v1.1$ alpha subunit protein are represented as relative expression to β -tubulin.

Mouse cortical tissues were dissected from animals at postnatal days (P) 1, 4, 7, 10, 14, 17, 21 and 30 to represent a wide range of developmental ages. Following membrane protein isolation as previously described (Jansen et al., 2010). Pelleted membrane proteins were resuspended in 1x RIA buffer (25 mM Tris, 150 mM NaCl, 1 mM EDTA, 2% Triton X-100, pH 7.4) and centrifuged at 20,000xg for 20 min, and the supernatant containing the membrane proteins was collected for analysis. Complete Protease Inhibitor set (Roche) was included in all solutions. Protein concentration was determined by BCA assay (Pierce) and transferred to nitrocellulose membranes and immunoblotted as previously described (Yu et al., 2003) with antibodies specific for $\text{Na}_v1.1$ (1/200, anti-rabbit $\text{Na}_v1.1$, Alomone labs), $\text{Na}_v1.3$ (1/100, Sp32iii, Westenbroek et al., 1992) and β -actin (1/10,000, anti-mouse beta actine, Abcam Inc.). Samples were background subtracted and are represented as relative expression to β -actin normalized to the maximum expression for each channel \pm SEM, n=3 for each time point represented.

Animal care and analysis

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Washington. *Scn1a*^{+/-} animals were maintained independently on both C57Bl/6 and 129SvJ backgrounds as described previously (Yu et al., 2006). Animals were genotyped using published primers. *Scn3a*^{+/-} animals were maintained on C57Bl/6 background and genotyped as previously described (Nassar et al., 2006). To generate double mutants, C57Bl/6 *Scn3a*^{+/-} females were crossed with *Scn1a*^{+/-} males on either C57Bl/6 or 129SvJ background to generate wildtype (WT), *Scn1a*^{+/-}, *Scn3a*^{+/-}, and *Scn1a*^{+/-}:*Scn3a*^{+/-} littermates. These F1 littermates were analyzed for both survival and thermal induction. Survival was assayed as the fraction of the starting population remaining alive over the course of 90 days after birth.

Thermal seizure induction

Each animal's core body temperature was continuously monitored by a rectal temperature probe and controlled by a feedback circuit in line with a heat lamp. Body temperature was increased in 0.5°C steps at 2 min intervals until a seizure occurred or a temperature of 42°C was reached. The animal was then cooled and returned to its home cage. Thermal induction was performed on postnatal day 21 (P21), and the induction process and resulting seizures were recorded by video monitoring. (Oakley et al., 2009).

<i>SCN1A</i> mutation	Number of cases	Age of onset (months)	Citation
+	18	5.6 ± 2.3	Petrelli et al, 2011
-	7	7.3 ± 4.2	
+	20	5.6 ± 2.1	Ragona, et al, 2011
-	6	5.8 ± 2.6	
+	7	5.7 ± 1.9	Chieffo, 2011
-	5	5.0 ± 1.1	
+	32	5.6 ± 2.1	Ragona, et al, 2010
-	5	6.2 ± 2.4	
+	29	5.7 ± 2.4	Lim, et al, 2011
Average onset:		5.7 ± 2.3	

+, indicates confirmed *SCN1A* mutation; -, denotes no *SCN1A* mutation detected

Table 4.1. Age of seizure onset in DS patients. Compilation of age of initial seizure onset in patients diagnosed with Dravet Syndrome. Studies were chosen that identified mutations present in *SCN1A* in addition to behavioral characteristics. Mutation positive (+) and mutation negative (-) were tabulated separately and reported. (Petrelli et al., 2011; Ragona et al., 2011; Ragona et al., 2010; Chieffo et al., 2011a; Lim et al., 2011)

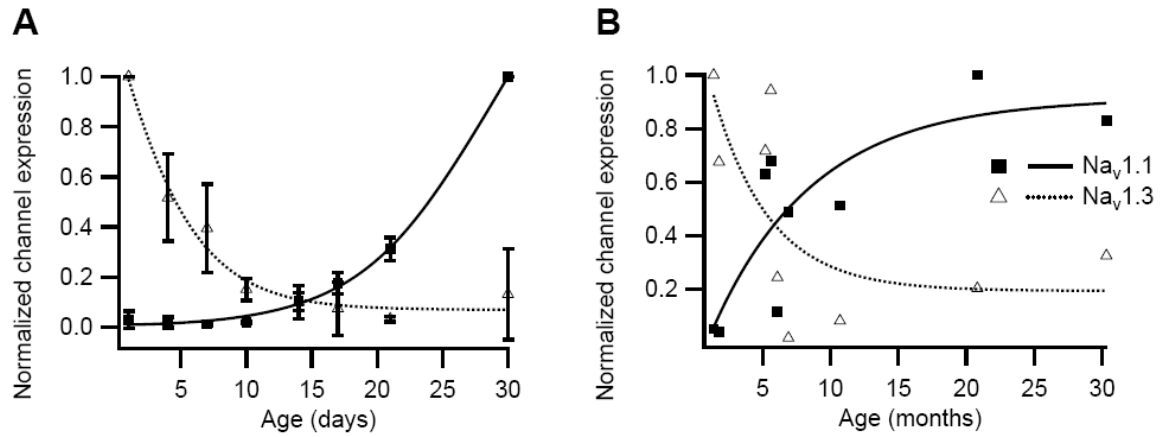


Figure 4.1. Expression of sodium channel proteins with respect to age in WT mouse and normal human cortex. (A) Channel expression in membrane proteins from normal C57Bl/6 mouse cortex. Samples are expressed as a fraction of max expression after normalizing to β -actin. Data points represented as mean \pm SEM. (B) Channel expression in membrane proteins from normal postmortem human cortical tissue. Samples are represented as a fraction of max expression after normalizing to β -tubulin.

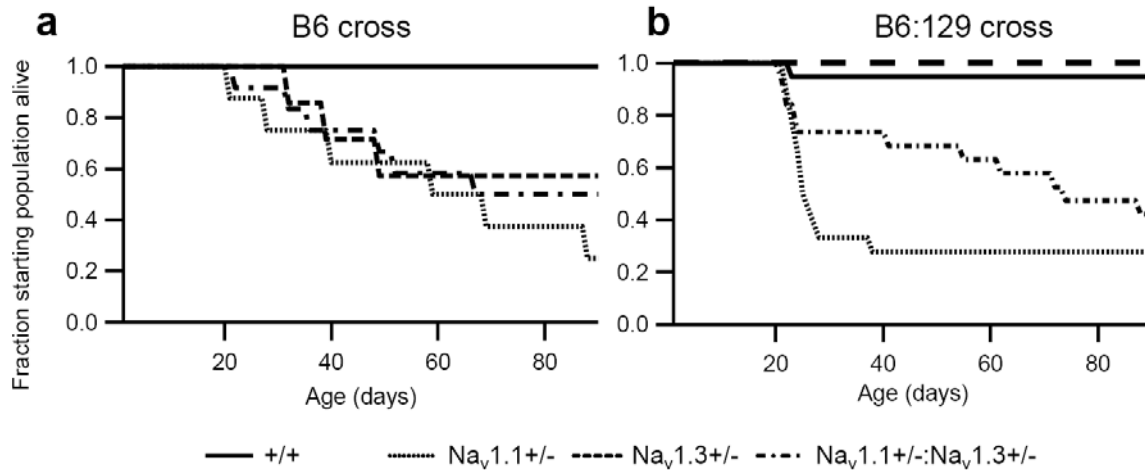


Figure 4.2. Survival of *Scn1a*^{+/-} animals is not impaired by loss of Nav1.3. (A) Survival of WT, *Scn1a*^{+/-}, *Scn3a*^{+/-}, and *Scn1a*^{+/-}:*Scn3a*^{+/-} double Het animals on C57Bl/6 background represented as the fraction of each genotypes surviving versus postnatal day (WT, n=10; *Scn1a*^{+/-}, n=8; *Scn3a*^{+/-}, n=6; *Scn1a*^{+/-}:*Scn3a*^{+/-} double Het, n=13). Differences between *Scn1a*^{+/-} and *Scn1a*^{+/-}:*Scn3a*^{+/-} survival are not statistically different at any age ($p > 0.05$) (B) Survival of WT, *Scn1a*^{+/-}, *Scn3a*^{+/-}, and *Scn1a*^{+/-}:*Scn3a*^{+/-} animals on a mixed C57Bl/6:129SvJ background represented as the fraction of each genotypes surviving versus postnatal day (WT, n=19; *Scn1a*^{+/-}, n=18; *Scn3a*^{+/-}, n=27; *Scn1a*^{+/-}:*Scn3a*^{+/-} double het, n=19). Differences between *Scn1a*^{+/-} and *Scn1a*^{+/-}:*Scn3a*^{+/-} populations are significantly different at P50 ($p < 0.05$) but not P90 ($p > 0.05$).

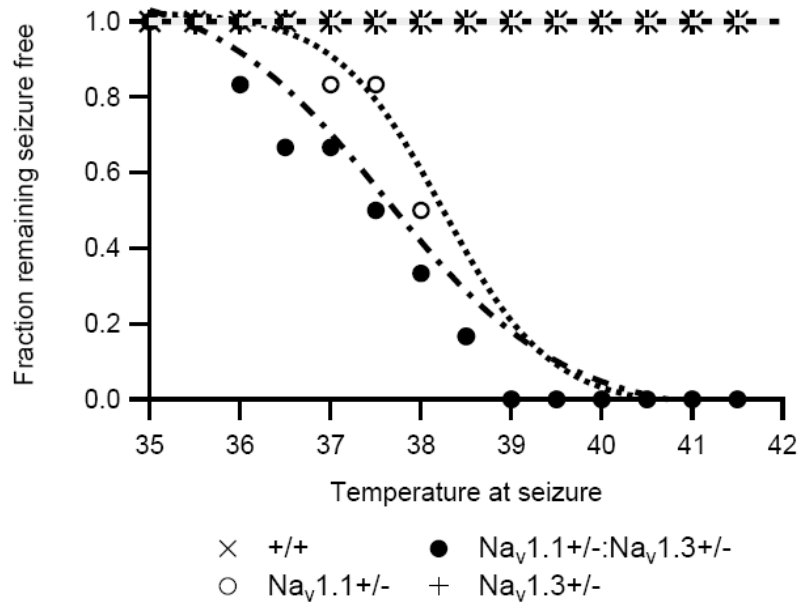


Figure 4.3. Sensitivity to thermally-induced seizures is unchanged in *Scn1a*^{+/-}:*Scn3a*^{+/-} compared to *Scn1a*^{+/-}. Thermally-induced seizures were evoked in all *Scn1a*^{+/-} and *Scn1a*^{+/-}:*Scn3a*^{+/-} animals with a mean seizure temperature of $38.7 \pm 0.4^\circ\text{C}$ and $38.1 \pm 0.5^\circ\text{C}$ respectively ($p > 0.05$). No WT or *Scn3a*^{+/-} mice presented with seizure. Data calculated as average \pm SEM (WT, n=14; *Scn1a*^{+/-}, n=6; *Scn3a*^{+/-}, n=12; *Scn1a*^{+/-}:*Scn3a*^{+/-} double Het, n=6).

Chapter 5 - Discussion & Conclusions

Mutations in sodium channel genes and dysfunction of the resulting proteins are often implicated in patients with epilepsy. Heterozygous loss-of-function mutations in *SCN1A* have frequently been associated with Dravet Syndrome (DS; Claes et al., 2001; Catterall et al., 2010). DS is a pharmaco-resistant infantile-onset epilepsy syndrome with co-morbidities including cognitive impairment, behavioral problems, motor impairment, and premature death. Several mouse models have been developed to study the role of sodium channel mutations in epilepsy and its comorbidities (Yu et al., 2006; Ogiwara et al., 2007; Martin et al., 2010a). This thesis has investigated the roles of two sodium channels, $Na_v1.1$ and $Na_v1.3$, in two separate mouse models of epilepsy. In a Cre-lox conditional heterozygous knockout mouse, one allele of $Na_v1.1$ was deleted solely in inhibitory interneurons and recapitulated the seizures and premature death seen in a global knockout mouse model of Dravet Syndrome (DS). Additionally, this conditional heterozygous knockout did not affect motor coordination but did impair social interactions and cognitive function. In a second global *Scn1a*^{+/-} mouse DS model, heterozygous global deletion of $Na_v1.3$ failed to exacerbate the symptoms of DS.

Conditional Loss of Na_v1.1 Solely in GABAergic Inhibitory Interneurons Leads to Seizures and Premature Death

In Chapter 2 of this thesis we used mouse genetics to delete Na_v1.1 exclusively in forebrain specific GABAergic inhibitory interneurons. Previous work had shown a specific loss of peak sodium current in inhibitory cells from global *Scn1a* mutant animals, and inhibitory neurons have been implicated by others in epilepsy pathogenesis (Cobos et al., 2005; Yu et al., 2006; Bender et al., 2012). To demonstrate a causal relationship between the two ideas, we generated a floxed *Scn1a* mouse that excises Exon-25 in the presence of Cre-recombinase (Appendix A). The specificity of our flox mouse was confirmed by western blot following a cross with an animal that globally expressed Cre recombinase. We chose to cross our flox mouse with a *Dlx1/2*-Cre animal that expresses Cre recombinase under the control of a *Dlx1* and *Dlx2* enhancer to specifically target GABAergic neurons in the forebrain. Through this cross we were able to functionally remove both alleles of Na_v1.1 in forebrain interneurons, and confirmed this deletion by immunohistochemistry.

After conditionally deleting Na_v1.1 in GABAergic forebrain interneurons, we found that heterozygous conditional mutants (*F/+;Dlx-Cre+*) animals replicated the spontaneous and temperature-induced GTC seizures seen in our global heterozygous *Scn1a* deletion (*Scn1a*^{+/}) model of DS. Spontaneous seizures in *F/+;Dlx-Cre+* animals began subtly and increased in both frequency and severity until animals progressed to generalized tonic-clonic seizures. Death followed these seizures 24-48 hours after initial seizure onset and this pattern was seen in all spontaneous deaths. In fact, no seizures were observed that did not progress to

death in this manner. These data clearly and directly implicate the dysfunction of forebrain interneurons due to the specific loss of Na_v1.1 in the seizures and death in mouse DS.

The approach that we have taken broadly targets interneurons based on their origins, and not on specific subclasses. Interneurons are a very diverse class of neurons essential for maintaining the balance of excitation and inhibition in the brain. These cells can be categorized into subclasses by considering their morphology, axonal connections, calcium binding protein expression, and firing patterns, and this variety is part of what makes the networks of inhibition and excitation in the brain complex (Markram et al., 2004; Ascoli et al., 2008). Further studies with more selective gene knockout methods and detailed physiological recordings are necessary to identify the specific neuron types whose loss of Na_v1.1 channels precipitates seizures and premature death.

Our conditional mutants had a high rate of premature death, which parallels the above-average incidence of sudden unexplained death in epilepsy (SUDEP) in DS patients (Genton et al., 2011; Sakauchi et al., 2011). While Na_v1.1 is primarily expressed in the brain, it is also expressed in the sino-atrial node, atria, and ventricles in the mouse heart (Maier et al., 2003). As would be expected in a forebrain specific conditional mutant, no deficits were found in the intrinsic cardiac function of F/+;Dlx-Cre⁺ animals when compared to their F/+;Dlx-Cre⁻ littermates. These results demonstrate that fatal dysfunction originates in the brain with the loss of Na_v1.1 channels in GABAergic interneurons and eliminates the loss of Na_v1.1 in spinal cord, peripheral nervous system, or heart as a requirement for premature death. However, further and more detailed studies are required to determine precisely

how loss of Na_v1.1 channels in GABAergic interneurons of the forebrain, and the resulting epileptic seizures, actually cause premature death. Disinhibition in specific brain regions could precipitate death from primary brain damage, respiratory failure, or heart failure caused indirectly by seizures.

Conditional Loss of Na_v1.1 Solely in GABAergic Inhibitory Interneurons Leads to Cognitive Impairment and Impaired Social Interactions, but not Ataxia

Chapter 3 of this thesis explored the role of GABAergic interneuron specific loss of Na_v1.1 in several comorbidities of DS. Individuals with DS have a number of comorbidities that can affect their quality of life (Brunklau et al., 2011). Many DS patients have varying degrees of cognitive and behavioral impairments, motor coordination difficulties, difficulty interacting with others, and often premature death following seizure onset (Wolff et al., 2006; Chieffo et al., 2011b; Genton et al., 2011; Guerrini and Falchi, 2011). Our *Scn1a*^{+/-} mutant mouse has already shown a mild ataxia and marked circadian rhythm disturbances (Kalume et al., 2007; Han et al., 2012). We used our F/+;Dlx-Cre+ animals to determine the role of Na_v1.1 channel loss in inhibitory interneurons on ataxia and cognitive function.

Gait analysis and tail suspension were utilized to determine the role of interneuron specific loss of Na_v1.1 on motor coordination. We found no differences in stride length or precision of forepaw/hindpaw placement between F/+;Dlx-Cre+ animals and F/+;Dlx-Cre- littermates. Similarly, no differences in full body spasticity were seen between the two

genotypes. When all measurements were repeated in global *Scn1a*^{+/-} DS mice on the same mixed CD1:C57Bl/6 background, no differences in motor coordination were observed between WT and heterozygous animals. These data are in contrast to the deficit in forepaw/hindpaw placement seen in pure strain C57Bl/6 *Scn1a*^{+/-} animals at the same age, suggesting genetic background may influence motor coordination (Kalume et al., 2007). Additionally, studies of human motor deficits in DS indicate that motor coordination impairments do not emerge until early adolescence, suggesting that future studies of motor coordination should be examined at an older age (Rodda et al., 2012).

Contextual fear conditioning was used to assay the effect of Na_v1.1 loss in interneurons on cognitive function. Our studies demonstrate that this cell-specific channel loss is sufficient to impair cognitive performance, suggesting that cognitive deficiencies in both humans and animal models are induced by reduced Na_v1.1 activity in forebrain interneurons. Other studies performed on these animals have also shown deficits in social interaction, hyperactivity and anxiety-like behavior, suggesting that Na_v1.1 activity in forebrain interneurons is also required for those neuropsychiatric functions as well (Han et al., 2012b).

Haploinsufficiency of Na_v1.3 does not Worsen Mouse DS

In Chapter 4, the role of Na_v1.3 as a potential genetic modifier and precipitating factor in DS was explored. We found the timing of Na_v1.1 expression closely paralleled the onset of

DS in humans, and seizures in *Scn1a*^{+/-} DS mice. These data in human and mouse suggest that the timing of DS onset is a result of the failure of Na_v1.1 to fully replace the loss of Na_v1.3. Thus, loss of more than 50% of Na_v1.3 channels coupled with failure of normal expression of Na_v1.1 channels contributes to the onset of symptoms in DS. Conversely, it is expected that increases in Na_v1.3 expression above 50% of its maximum expression level may prolong the onset of DS and could be an interesting therapeutic target in future studies.

To assess potential compensation from Na_v1.3 in the survival of DS animals, a double genetic deletion was performed by crossing a mutant Na_v1.3 animal with our DS animal. Exons 4 and 5 of *Scn3a*, the gene encoding Na_v1.3, were deleted to produce a Na_v1.3 heterozygous animal (*Scn3a*^{+/-}; Nassar et al., 2006). If Na_v1.3 is aiding in the survival of *Scn1a*^{+/-} DS mice, as suggested previously, survival of *Scn1a*^{+/-}:*Scn3a*^{+/-} double hets should be worse when compared to survival of DS mice alone. We found survival of *Scn1a*^{+/-}:*Scn3a*^{+/-} double Het mice to be no more impaired than in *Scn1a*^{+/-} alone in both pure C57BL/6 and mixed 129SvJ:C57Bl/6 backgrounds. These results show that loss of one allele of *Scn3a*, has no detrimental impact on survival in DS mice. Similarly, we assayed the sensitivity of *Scn1a*^{+/-}:*Scn3a*^{+/-} double Het animals to thermally induced seizures and found them to be equally susceptible as their *Scn1a*^{+/-} littermates. These data suggested that haploinsufficiency of Na_v1.3 channels is not sufficient to exacerbate sensitivity to thermally induced seizures.

Our results from two different experimental approaches shed new light on the possibility that the Na_v1.3 channel may serve as a precipitating factor or genetic modifier in DS. This work aligns with our previous observation that Na_v1.3 channels are up-regulated in the hippocampus of *Scn1a*^{+/-} mice (Yu et al., 2006). Considered together, an important implication of these results is that up-regulation or prolongation of expression of Na_v1.3 channels to levels approaching 50% of their maximum could potentially blunt the adverse effects of loss of Na_v1.1 channels in DS.

Conclusions

Altogether, these data continue to expand our knowledge of the role of sodium channels in epilepsy. We now know that loss of Na_v1.1 solely in inhibitory interneurons of the forebrain can recapitulate the core DS phenotype and impair cognitive function. This opens the door to better understand the role of both Na_v1.1 and interneurons in other DS comorbidities and potential therapeutic strategies. Additionally, our floxed *Scn1a* mouse will undoubtedly be a valuable tool moving forward as we probe and expand our understanding of the role of Na_v1.1 in different neural circuits and their associated behaviors and phenotypes.

Additionally, we have expanded our knowledge of the interplay between Na_v1.1 and Na_v1.3 in DS development. Our studies have shown that loss of Na_v1.3 below 50% of its maximum level coincides with the onset of DS and may be a precipitating factor in the disease. We have also found is that additional loss of Na_v1.3 channels does not exacerbate

the DS phenotypes. However, expression at this low level leaves the door open to consider a potential therapeutic role for $\text{Na}_v1.3$ if its expression can be increased or prolonged to above 50% of its maximum expression.

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Appendix A - Generation of Na_v1.1 Flox animal

This work was completed by Dr. Frank H. Yu, currently of the School of Dentistry, Seoul National University and is presented here as a consolidated resource and reference for our research group.

All animal procedures were approved by the Institution Animal Care and Use Committee and the University of Washington. To generate a floxed Na_v1.1 animal, Exon-25, the second to last coding exon of *Scn1a*, was cloned into plasmid 4517D (a gift from Stan McKnight) already containing a single LoxP site, FRT-flanked neomycin selection cassette, and multiple cloning sites (Figure A1). The Nhe/Sal sites were used to clone in a 6 kb long arm containing Exon26 (DIV S3-CT), and the Xho/Not sites were used to clone in a 1.5 kb short arm containing exon25 (DIV S1-S3) and a second loxP site. The plasmid was linearized at the Asc1 site and electroporated into embryonic stem cells and used to generate chimeric mice (Figure A2).

Following germline transmission, neomycin-containing flox mice were analyzed for brain Na_v1.1 protein expression by western blot. Na_v1.1 protein expression in flox-containing samples was reduced when compared to WT (non-flox containing) animals (Figure A3). As a result, the neomycin selection cassette was removed by mating with a flipase expressing mouse (B6.SJL-Tg(ACTFLPe)9205Dym/J, Jackson Labs) to avoid possible impairment of normal *Scn1a* expression. The resulting neomycin excised animals were backcrossed with C57BL/6J (Jackson Labs) for 10 generations and used in all subsequent experiments.

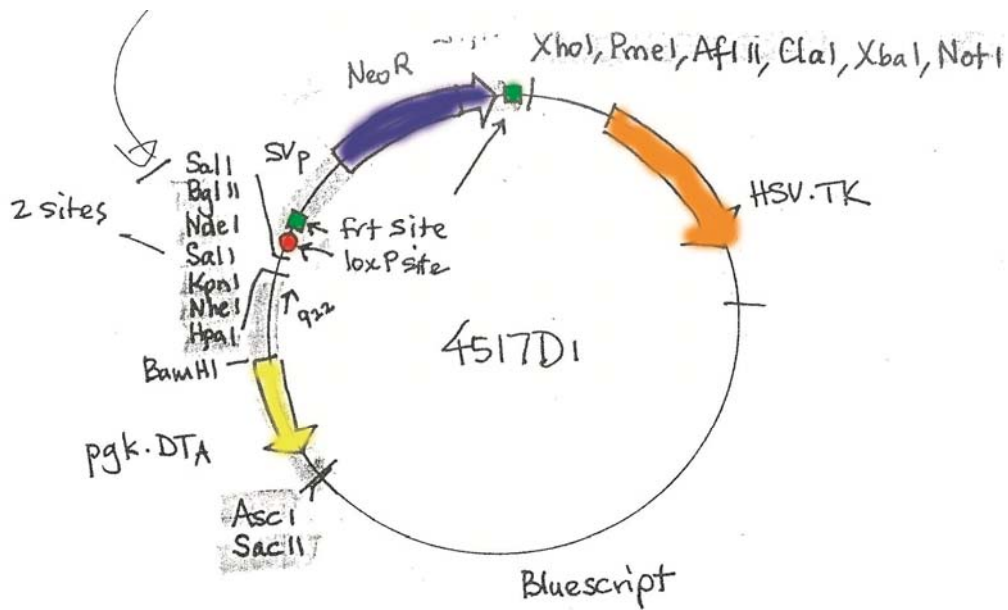


Figure A1. Vector schematic of 4517D1 used to generate the floxed *Scn1a* gene. At left the *Nhe*/*Sal*I sites were used to insert the 6kb long arm of the targeting construct containing Exon 26 (DIV S3-CT). At top of the vector the *Xho*/*Not*I sites were used to clone the 1.5 kb short arm of the targeting vector containing Exon25 (DIV S1-S3) and the second loxP site.

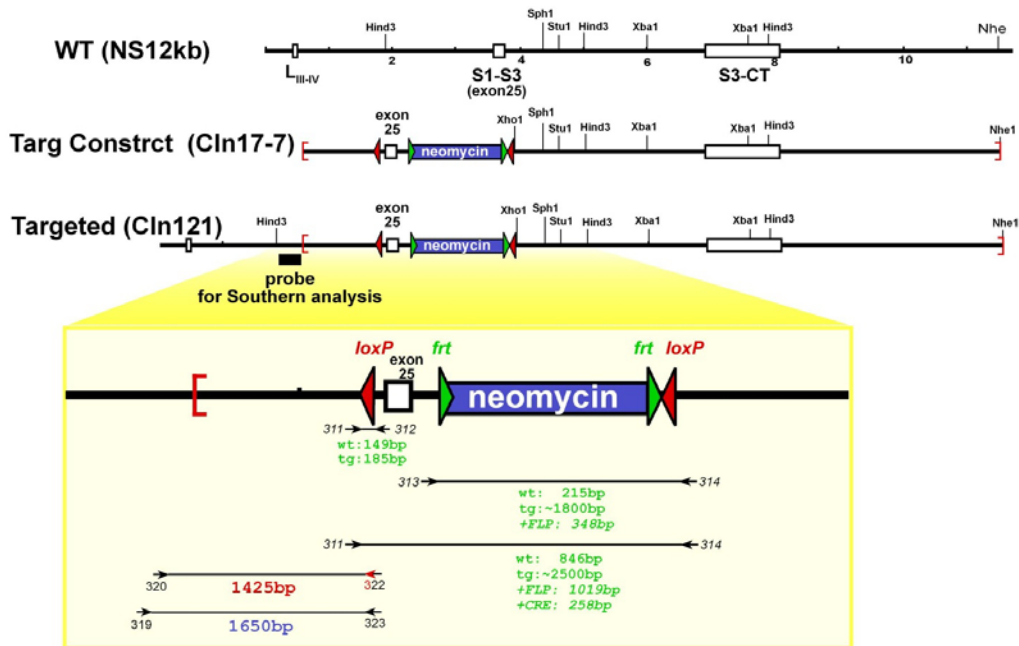


Figure A2. Schematic representation of WT, targeting construct, and targeted DNA. *Top*, WT genomic DNA representing Exon25 and Exon26. *Middle*, targeting construct as it appears in the vector 4517D after the introduction of Exon 25 (with the additional loxP site) and Exon 26 on either side of the existing neomycin selection cassette. *Bottom*, following homologous recombination the targeting construct is integrated into the genomic DNA of chimeric animals generating the floxed *Scn1a* animal. *Insert*, location of primers for genotyping. Animals were genotyped using primers FHY311 (5'-CTTGATGTGTTGAAATTCAC-3') and FHY314 (5'-TATAGAGTGTTAATCTCAAC-3'). WT allele: 846 bp, Flox allele: 1019 bp, Excised allele: 258 bp

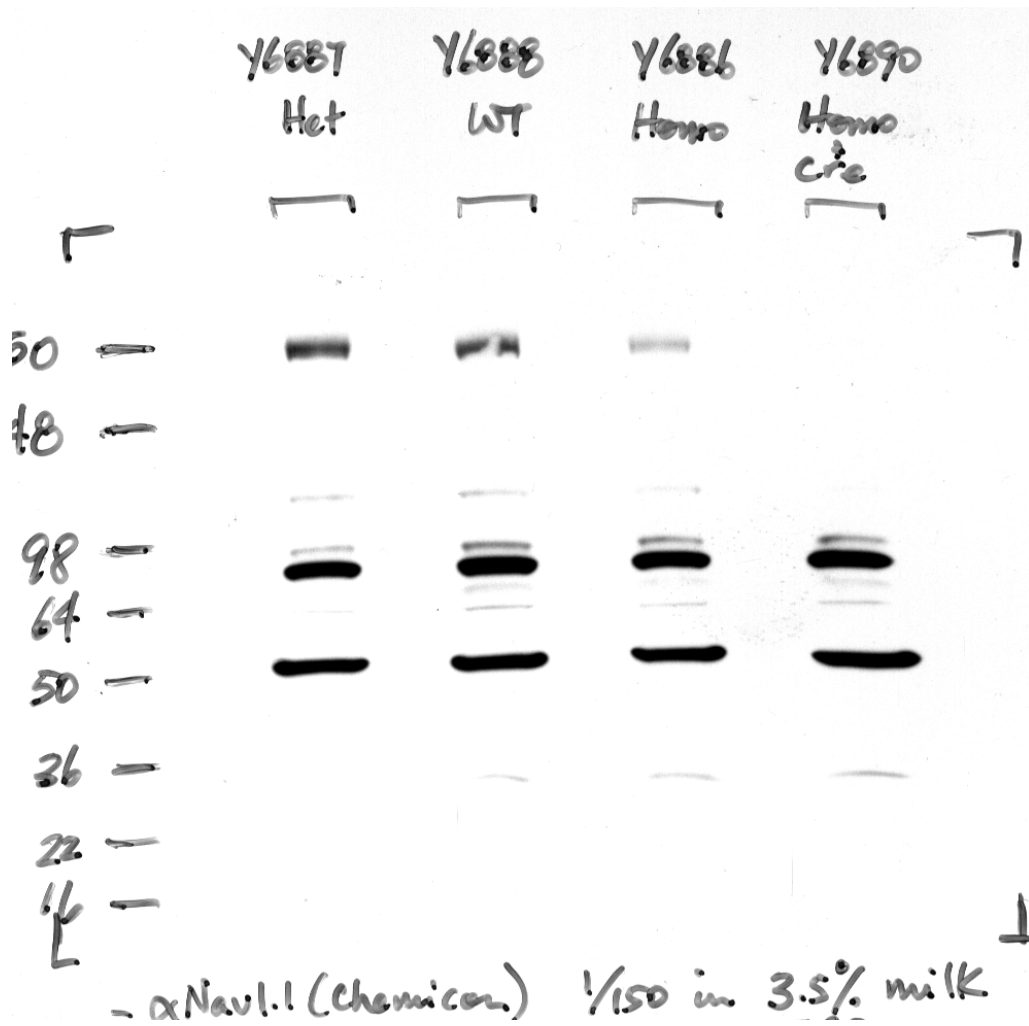


Figure A3. Na_v1.1 expression is hypomorphic in the presence of neomycin selection marker. Western blot of membrane proteins from global Het, WT, homozygous flox and homozygous flox in the presence of Meox-Cre. Top band at ~250kDa is specific for Na_v1.1, lower molecular weight nonspecific bands serve as a protein loading control. (Het, global Nav1.1 Het; WT, wildtype animal; Homo, homozygous flox allele (F/F); Homo+Cre, homozygous flox (F/F) in the presence of a globally deleting Meox-Cre)

Appendix B - Measurements of Na_v1.1 and Na_v1.3 protein and RNA expression in global *Scn1a*^{+/-} DS mice

Compensating increases in sodium channel Na_v1.3 following loss of sodium channel Na_v1.1 was introduced and explored in Chapters 1 and 4 respectively. Previous work in *Scn1a* mutant mice in mixed background (50:50, 129SvJ:C57BL/6) has shown increased Na_v1.3 expression in *Scn1a*^{-/-} or *Scn1a*^{+/-} animals compared to wildtype. However, these elevated levels of Na_v1.3 protein were still insufficient to prevent death in *Scn1a*^{-/-} mice (Yu et al., 2006) We suggested that increases in Na_v1.3 expression may contribute to the mixed rate of death seen in animals on the 50:50 strain, and therefore that *Scn3a* may be an attractive genetic modifier gene that may convey increased survival/decreased death when separated onto pure strains. To investigate this hypothesis, protein expression of Nav1.3 was quantified using immunohistochemistry (IHC) and mRNA levels of *Scn3a*, the gene encoding Na_v1.3, were measured using quantitative real-time PCR (qRT-PCR) in *Scn1a*^{+/-} animals on C57BL/6 and 129SvJ backgrounds.

We anticipated that the seizure-resistant 129SvJ *Scn1a*^{+/-} animals would have more Na_v1.3 protein in hippocampal GABAergic interneurons and elevated expression of *Scn3a* mRNA than their susceptible C57BL/6 counterparts. In practice, we found a large animal to animal biologic variability that made it difficult to draw clear conclusions.

Immunostaining of WT and *Scn1a*^{+/-} mice on C57Bl/6 and 129SvJ backgrounds for Na_v1.3

Na_v1.3 positive interneurons in P14 animals were counted after immuno-labeling with antibodies specific for Na_v1.3 and GAD67. In the original experiments, differences between WT, *Scn1a*^{+/-}, and *Scn1a*^{-/-} animals were seen at P14. Therefore, IHC experiments were performed on tissue from P14 WT and *Scn1a*^{+/-} animals on C57Bl/6 and 129SvJ backgrounds. Data from this age produced widely varied results and failed to show statistically significant differences between wildtype and *Scn1a*^{+/-} 129SvJ animals when compared to C57Bl/6 animals of the same genotypes (Fig. B1). In some individual experiments there were clear differences in Na_v1.3 expression levels between genotypes and mouse strains, however, when averaged together these differences were obscured.

In our analysis, when a cell stains for GAD67 and Na_v1.3, it is counted as positive regardless of the intensity of antibody labeling. It is possible that lack of statistically significant differences between the wildtype and *Scn1a*^{+/-} levels of each mouse strain is due to difficulty distinguishing staining intensity between wildtype and heterozygous *Scn1a*^{+/-} samples. But a greater problem was the huge biologic variability in staining between animals.

Quantification *Scn3a* mRNA levels by qPCR in WT and *Scn1a*^{+/-} mice on C57Bl/6 and 129SvJ backgrounds

The levels of Na_v1.3 mRNA in the hippocampi of *Scn1a*^{+/-} and wildtype 129SvJ and C57BL/6 animals were compared using SYBR green qRT-PCR at postnatal days 14, 21 and 28 (P14, P21, P28). Time points were chosen based on rates of animal survival. At P14 the knockout animals on all backgrounds die, but at this age *Scn1a*^{+/-} animals on C57BL/6 and 129SvJ backgrounds are phenotypically indistinguishable from their WT littermates. By P21, C57BL/6 *Scn1a*^{+/-} animals begin to die, have spontaneous seizures and become susceptible to thermally induced seizures (Yu et al., 2006; Oakley et al., 2009). By P28, death continues in C57Bl/6 *Scn1a*^{+/-} animals while 129SvJ *Scn1a*^{+/-} animals have not. Wildtype survival is unaffected throughout.

Average data at P14 showed a statistically significant increase in *Scn3a* mRNA levels between 129SvJ *Scn1a*^{+/-} samples and C57BL/6 WT (Fig. B2A). At P21, a statistically significant increase in *Scn3a* mRNA was observed in 129SvJ WT animals when compared to C57BL/6 WT animals. The apparent increase in *Scn3a* mRNA in 129SvJ *Scn1a*^{+/-} animals was not found to be significant when compared to C57BL/6 animals of the same genotype, or WT animals on either strain (Fig. B2B). The most interesting results come at P28, where 129SvJ WT and *Scn1a*^{+/-} samples have significantly increased levels of *Scn3a* when compared to their C57BL/6 counterparts and were significantly different from each other (Fig. B2C). These data, while exciting and statistically significant, still have a large biologic variability in which 2 experiments seem to contain animals that express *Scn3a* at very high levels relative

to the others tested. This variability is likely due to individual animal variability as seen in the IHC data. Some of the noise could potentially be reduced by repeating the experiments with taqman primers.

Discussion

One possible interpretation of these results is that decreased $\text{Na}_v1.1$ protein expression prolongs the decline of $\text{Na}_v1.3$ and this higher level of $\text{Na}_v1.3$ expression may be responsible for the individual variability in seizure onset and/or death in our $\text{Scn1a}^{+/-}$ DS model. The biologic variability seen in our IHC and qPCR data may indicate individual differences in $\text{Na}_v1.3$ expression within a strain are equally as important as differences in channel expression between strains. Death is not a coordinated event in $\text{Scn1a}^{+/-}$ animals on both strains and some heterozygous animals can live for months or years, suggesting that individual genetic changes or environmental effects play a significant role in animal survival. If changes in $\text{Na}_v1.3$ expression in individual animals are responsible for variable rates of survival and death, it is impossible to separate animals that would have lived from animals that would have died at any time prior to death. Notably, the most promising results came from the qPCR data at P28. At this age a number of $\text{Scn1a}^{+/-}$ mice have died and are no longer in our experimental population. Repeating these experiments in older animals, >90 days, could give a more definitive answer and should result in a much tighter data set.

However, changes in *Scn3a* mRNA levels do not directly translate into changes in Na_v1.3 protein levels and a more definitive approach would be to use mouse genetics to force the over expression of Na_v1.3 and observe its effects on survival and other DS phenotypes. If Na_v1.3 performs a redundant role with Na_v1.1, it should be able to replace the missing channel and we would expect a milder DS phenotype in the resultant animals. The data from chapter 4 showed that the postnatal expression of Na_v1.3 is at low levels and that there is room for increases in expression, but likely these would have to be over 50% of the maximum expression to see an effect.

Methods

Immunohistochemistry

Animals were perfused at P14 with 4% paraformaldehyde in PBS, brains were removed and saturated in 30% sucrose, sliced to 50 μm, and double-labeled as free-floating slices (Westenbroek et al., 1998). Primary antibodies were affinity-purified rabbit anti-Na_v1.3 (1:15, Alomone) and mouse anti-GAD67 (1:400, Millipore). Secondary antibodies were goat anti-mouse IgG labeled with Alexa555 (1:400, Invitrogen), goat anti-rabbit IgG labeled with Alexa488 (1:400, Invitrogen). Tissue samples from WT and *Scn1a*^{+/-} animals on both 129SvJ and C57Bl/6 backgrounds were processed simultaneously. Gain- and offset-matched images were collected on a Leica SL confocal microscope at the Keck Imaging Facility of the University of Washington. Sections stained in the absence of primary antibody showed no

detectable labeling. Separate fluorescence channel images of each antibody were analyzed and positively labeled cells were marked using Adobe Photoshop (Adobe Creative Suite, v 10.0.1). Then images from the two channels were compared and cells that were clearly stained for both were counted as colocalized.

Quantitative Real-Time PCR (qPCR)

Hippocampal tissue was isolated from P14, P21 or P28 mice, snap frozen with liquid nitrogen and kept frozen at -80°C until enough tissue was ready for experiments. RNA isolated with RNeasy kit (Qiagen) following homogenization with the QiaShredder column (Qiagen) and was stored at -20°C until needed. Primer pairs specific for Na_v1.3 and acidic ribosomal binding protein (ARBP; Table B1) were used in parallel and treated with a reverse transcriptase to generate cDNA in the PCR reaction tube prior to starting SYBR green visualized PCR. Each sample was run in triplicate and a Na_v1.3 specific no RT negative control was included. The samples were prepared in the proportions dictated by Stratagene using their SYBR green fluorescent dye. Samples were run on Stratagene's Mx3000P machine using a two-step temperature protocol. Data were quantified using the $\Delta\Delta C_t$ method and within an experiment data were normalized to C57BL/6 WT values. Average data in Figure B2 represents the average for each genotype from individual experiments. N values are a representation of separate experiments. P values were determined using two-tailed, unpaired t-test.

	PrimerBank ID	Primer ID		Sequence	length	Tm	Location
Nav1.3	9055330a3	CC-5	Forward	CCTTAAAGACGGTAACGGAACC	22	61	585-606
		CC-6	Reverse	GGTGGCCTGAACTGAAAAGC	20	62	817-798
ARBP	6671569a3	ARBP forward	Forward	TGTTTGACAACGGCAGCATT	21	62	575-595
		ARBP reverse	Reverse	CCGAGGCAACAGTTGGGTA	19	62	709-691

Table B1. Primers used of qPCR. Validated primer sequences for Nav_v1.3 and ARBP were obtained from the Harvard primer bank (<http://pga.mgh.harvard.edu/primerbank/>). Working stock solutions were diluted to 10μM and stored at -20°C.

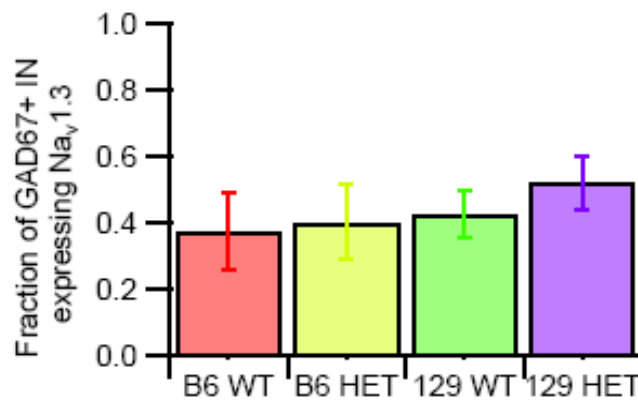


Figure B1. No differences were seen in Nav1.3 expression in GAD67 positive interneurons of the hippocampus between WT and *Scn1a*^{+/-} animals on C57Bl/6 and 129SvJ backgrounds. Sections of P14 tissue from C57Bl/6 WT, C57Bl/6 *Scn1a*^{+/-}, 129SvJ WT, and 129SvJ *Scn1a*^{+/-} were double labeled with antibodies for Nav_v1.3 and GAD67. The number of Nav_v1.3 positive, GAD67 positive and Nav_v1.3/GAD67 double labeled neurons were counted and are represented as a fraction of total GAD67 positive interneurons in a sample. Total numbers of neurons were counted in CA1, CA3 and DG regions of four tissue slices and the average was represented in the bar graph for each genotype/strain combination. No statistically significant differences were seen between any genotype or strain. (C57Bl/6 WT, .32 ± .12; C57Bl/6 *Scn1a*^{+/-}, .40 ± .11; 129SvJ WT, .43 ± .07; 129SvJ *Scn1a*^{+/-}, .52 ± .08; N=5 experiments)

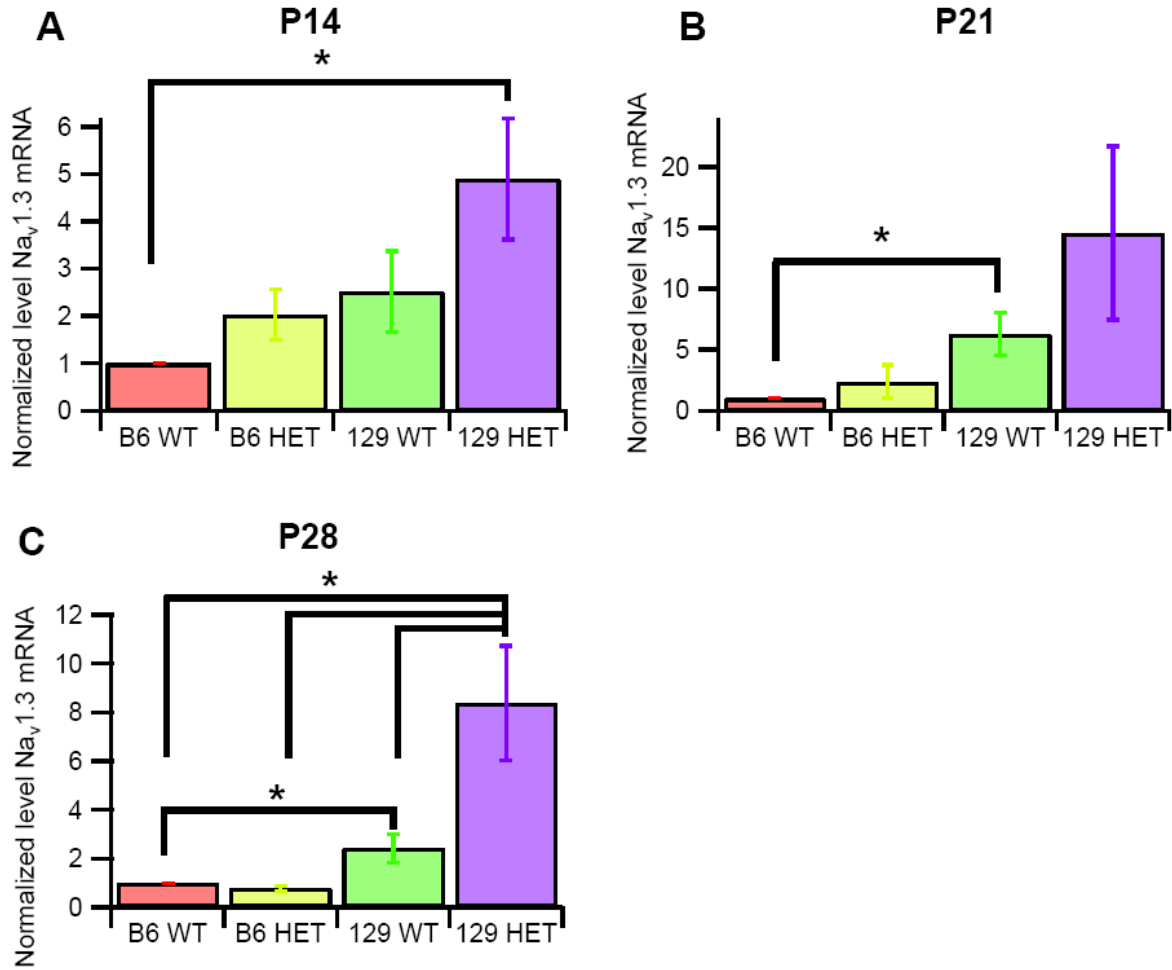


Figure B2. mRNA levels of *Scn3a* in WT and *Scn1a*^{+/-} animals on C57Bl/6 and 129SvJ genetic backgrounds. Individual experiments were normalized to C57Bl/6, WT *Scn3a* expression levels from that experiment. Averages were then taken of those relative amounts and represented here. **(A)** At P14, *Scn3a* mRNA levels appear elevated in the 129SvJ *Scn1a*^{+/-} sample, but the only statistically significant difference is between 129SvJ *Scn1a*^{+/-} and C57Bl/6 WT (N=6; C57Bl/6 *Scn1a*^{+/-}, 2.0 ± 0.5; 129SvJ WT, 2.5 ± 0.9; 129SvJ *Scn1a*^{+/-}, 4.9 ± 1.3). **(B)** At P21, *Scn3a* mRNA levels appear elevated in the 129SvJ *Scn1a*^{+/-} sample, but the high rate of biologic variability renders those differences insignificant. The only statistically significant difference is between C57Bl/6 and 129SvJ WT animals (N=11; C57Bl/6 *Scn1a*^{+/-}, 2.4 ± 1.4; 129SvJ WT, 6.3 ± 1.8; 129SvJ *Scn1a*^{+/-}, 14.8 ± 7.1). **(C)** At P28, *Scn3a* mRNA levels are elevated in the 129SvJ *Scn1a*^{+/-} sample set and are statistically higher than C57Bl/6 *Scn1a*^{+/-}, C57Bl/6 and 129SvJ WT samples. Significant differences were also seen between WT samples on both strains (N=6; C57Bl/6 *Scn1a*^{+/-}, 0.78 ± 0.1; 129SvJ WT, 2.4 ± 0.6; 129SvJ *Scn1a*^{+/-}, 8.4 ± 2.3). * p<.05

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2004-2012	<i>University of Washington</i> Department of Pharmacology Doctor of Philosophy	<i>Seattle, WA</i>
2000-2004	<i>The Ohio State University</i> Bachelor of Science, Chemistry	<i>Columbus, OH</i>
2000-2004	<i>The Ohio State University</i> Bachelor of Science, Molecular Genetics	<i>Columbus, OH</i>

Honors/Awards:

Drug Action, Metabolism and Kinetics Training Grant (NIH 5T32 GM007750-30)	2005-2008
Recipient of Graduate School Fund for Excellence and Innovation Merit Award	2004
Recipient of Battelle Memorial Institute Leaders of Tomorrow Scholarship	2000-2004

Research Experience:

University of Washington, Dept. of Pharmacology, Seattle, WA

PhD Candidate

(July 2004 – June 2012)

- Studying the role of sodium channel Na_v1.1 in epilepsy, primarily utilizing the Cre-loxP system to target deletion specifically to interneurons. Other aspects of this research have included using molecular biology, biochemistry, immunohistochemistry, animal behavior and mouse genetics to study other aspects of mouse models of epilepsy.
- Advisor: William A Catterall, PhD

The Ohio State University Dept. of Medicinal Chemistry, Columbus, OH
Undergraduate Research Assistant (March 2003 – June 2004)

- Research focused on testing anti-proliferative, and anti-tubulin polymerization agents on several cancer cell lines and elucidating the apoptosis pathways in addition to cell line and general lab maintenance. Techniques involved included tissue culture and biochemistry (western blotting, ELISA assays, etc.)
- Advisor: Pui-Kai Li, PhD

The Ohio State University Dept. of Medicinal Chemistry, Columbus, OH
Undergraduate Research Assistant (June 2002 – November 2002)

- Responsibilities included creating new PC3 cell lines for use in anti-cancer drug screening.
- Advisor: Ching-Shih Chen, PhD

The James Cancer Research Institute Bone Marrow Transplant Lab, Columbus, OH
Undergraduate Student Lab Research Volunteer (September 2001 – June 2002)

- Laboratory Prep work, and assistance with mini-preps, RNA and DNA extractions and other basic molecular biology required for the lab's genetic leukemia research.
- Advisor: Belinda R Avalos, MD

Teaching Experience:

University of Washington, Department of Pharmacology, Seattle, WA
Teaching Assistant (September 2005-March 2006)

- Managing and running a formal discussion session for Pharmacy students enrolled in Pharmacology 401 and 402.
- Created and graded weekly quizzes
- Evaluated and graded exams

Publications:

Cheah CS, Westenbroek RE, Roden WH, Oakley JC, Jansen LA, Wood J, Catterall WA. Sodium Channel Nav1.3 as a potential precipitating factor and genetic modifies in Dravet Syndrome. (2012) *In preparation*

Kalume F, Westenbroek RE, **Cheah CS**, Oakley JC, Scheuer T, Catterall WA. Mechanism of Sudden Unexpected Death in a Mouse Model of Dravet Syndrome. (2012) *submitted*

Cheah CS, Yu FH, Westenbroek RE, Kalume FK, Oakley JC, Potter GB, Rubenstein JL, Catterall WA. Specific Deletion of Nav1.1 Channels in Inhibitory Interneurons Causes Seizures and Premature Death in a Mouse Model of Dravet Syndrome. (2012) *In press, PNAS*

Kwan W, Traeger U, Davalos D, Chou A, Andre R, Miller A, **Cheah C**, Moeller T, Stella N, Akassoglou K, Tabrizia S, Muchowski P. Mutant huntingtin impairs the migration of immune cells in Huntington disease. (2012) *Accepted, Journal of Clinical Investigation*

Han S, Tai C, Westenbroek RE, Yu FH, **Cheah CS**, Potter GB, Rubenstein JL, Scheuer T, de la Iglesia HO, Catterall WA. Autism-related behaviors and cognitive deficit in a mouse model of Dravet Syndrome and rescue by enhancing inhibitory neurotransmission. (2012) *Accepted, Nature*

Giorgini F, Möller T, Kwan W, Zwilling D, Wacker JL, Hong S, Tsai LC, **Cheah CS**, Schwarcz R, Guidetti P, Muchowski PJ. Histone deacetylase inhibition modulates kynurenine pathway activation in yeast, microglia, and mice expressing a mutant huntingtin fragment. *J Biol Chem*. 2008 Mar 21;283(12):7390-400.

Newhall KJ, Criniti AR, **Cheah CS**, Smith KC, Kafer KE, Burkart AD, McKnight GS. Dynamic anchoring of PKA is essential during oocyte maturation. *Curr Biol*. 2006 Feb 7;16(3):321-7.

Abstracts:

Cheah C S, Yu FH, Westenbroek RE, Kalume FK, Oakley JC, Rubenstein JL, Catterall WA. Conditional deletion of NaV1.1 channels in inhibitory interneurons is sufficient to cause the seizures and premature death in a mouse model of SMEI. *Neuroscience Abstracts*255.216 San Diego, CA, 2010

Kwan W, Giorgini F, **Cheah C**, Miller A, Stella N, Moller T, Guidetti P, Schwarcz R, Muchowski PJ. Mutant huntingtin in microglia may contribute to neuronal dysfunction in Huntington's disease. Society for Neuroscience, San Diego, CA, 2007.