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Nikki T. Sawyer Date

The Role of Stress in Two Genetic Models of Epilepsy

By

Nikki T. Sawyer

Doctor of Philosophy

Graduate Division of Biological and Biomedical Science

Neuroscience

_____	_____
Andrew Escayg, Ph.D. Advisor	Gretchen Neigh, Ph.D. Advisor
_____	_____
Tamara Caspary, Ph.D. Committee Member	Mar Sanchez, Ph.D. Committee Member

_____

David Weinshenker, Ph.D.
Committee Member

Accepted:

_____

Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

_____

Date

The Role of Stress in Two Genetic Models of Epilepsy

By

Nikki T. Sawyer

B.S., Clayton State University, 2007

Advisors: Andrew Escayg, Ph.D. and Gretchen Neigh, Ph.D.

An abstract of
a dissertation submitted to the Faculty of the
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Abstract

The Role of Stress in Two Genetic Models of Epilepsy
By Nikki T. Sawyer

Human studies show a link between stress and epilepsy, with stress causing an increase in seizure frequency and severity in patients with epilepsy. While many different animal model systems have been used to better understand this connection and the possible mechanisms involved, results have not been clear. Furthermore, little is currently known about how a genetic predisposition to epilepsy interacts with the stress response to influence seizure outcome. To address this question, we examined the relationship between stress and seizure outcome in two genetic models of epilepsy that carry mutations in voltage-gated sodium channels (VGSCs) *Scn8a* and *Scn1a*. *Scn8a*$^{med/+}$ mutants display spontaneous absence seizures and an increased threshold to induced seizures. *Scn1a*$^{RH/+}$ mutants exhibit spontaneous convulsive seizures and a reduced threshold to induced seizures. In the experiments described herein we demonstrate that the VGSC mutations can affect the stress response, seizure outcomes following either a stressor or altered early life experience, and behavior. *Scn8a*$^{med/+}$ mutants respond to stress differently from their wildtype (WT) littermates, and higher levels of anxiety-like behaviors may be driving the seizure response to a stressor in this model. Stress also negatively affects the *Scn1a*$^{RH/+}$ mutants, but in a similar manner to their WT littermates. The *Scn8a*$^{med/+}$ line and *Scn1a*$^{RH/+}$ line are maintained on different mouse strains, suggesting that genetic background and the presence of modifier genes may also affect the seizure response to a stressor. We also demonstrate that experiences encountered early in life can modify seizure outcome in adult animals in ways that depend on both the genetic makeup of the organism and sex of the organism. Overall, we show that altered neural excitability due to an epilepsy-causing mutation can affect the stress response, seizure outcomes following either a stressor or altered early life experience, and behavior. These results highlight the importance of using genetic models of epilepsy to better understand how stress is working to influence seizure activity.

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mentor someone else's scientific journey, and I look forward to helping others realize their scientific dreams in the future. I must also thank Stacey B.B. Dutton for her encouragement and advice. As a Neuroscience Program graduate student two years ahead of me, she provided loads of helpful hints and advice for surviving graduate school and she helped to keep me sane. I would like to also thank Chris Makinson, a fellow Neuroscience Program graduate student that started the same time as me, who provided moral support and guidance and gave me the comfort that someone else was frequently in the same boat I was! I also want to thank Tyra Lamar for her constant optimism, joyful spirit, and upbeat personality. Tyra helped me to renew my love for science at a time when I needed it the most. I also want to particularly thank some of the students from the Weinshenker lab for their invaluable support, encouragement, and advice that was truly appreciated over the years: Alisha Epps, Meriem Gaval, and Heather Mitchell. There are many other people who have helped to shape my life and career over the past six and a half years, and I would like to thank everyone who has had a hand in this incredible journey. If I failed to acknowledge you here, it does not mean that I am not thankful for your help and support.

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The journey of our lives is not just about the destinations we have reached. Our wisdom, education, and personal growth come from the people we meet, the people we love, the paths we choose to follow, and the lessons we have learned along the way.

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Abbreviations

4-AP	4-aminopyridine
ACTH	Adrenocorticotropic Hormone
ADHD	Attention Deficit Hyperactivity Disorder
ANOVA	Analysis of Variance
ASD	Autism Spectrum Disorder
BNST	Bed Nucleus of the Stria Terminalis
CORT	Corticosterone
CRH	Corticotropin Releasing Hormone
CS	Conditioned Stimulus
DH	Daily Handled
DS	Dravet Syndrome
EEG	Electroencephalogram
EL	Epilepsy (Mouse)
EMG	Electromyography
GABA	γ-aminobutyric Acid
$GABA_A$	γ-aminobutyric Acid Subtype A (Receptor)
GAERS	Genetic Absence Epilepsy Rats from Strasbourg
GEFS+	Genetic Epilepsy with Febrile Seizures Plus
GnRH	Gonadotropin Releasing Hormone
GR	Glucocorticoid Receptor
GTCS	Generalized Clonic-Tonic Seizure
HPA	Hypothalamic-Pituitary-Adrenal (Axis)
HPF	Highly Palatable Food
IACUC	Institutional Animal Care and Use Committee
i.c.v.	Intracerebroventricular
i.p.	Intraperitoneal
Iso	Isoniazid
KA	Kainic Acid
MJ	Myoclonic Jerk
MR	Mineralocorticoid Receptor
MS	Maternal Separation
Picro	Picrotoxin
PPI	Prepulse Inhibition
PR	Post-Restraint
PTZ	Pentylenetetrazol
PV	Parvalbumin
PVN	Paraventricular Nucleus
RIA	Radioimmunoassay

SAM	Sympathetic-Adrenomedullary (System)
SCF	Standard Chow Food
SE	Status Epilepticus
SEM	Standard Error of the Mean
SUDEP	Sudden Unexplained Death in Epilepsy
SWD	Spike-Wave Discharge
THDOC	Allotetrahydrodeoxycorticosterone
Thio	Thiosemicarbazide
TMT	2,5-dihydro-2,4,5-trimethylthiazoline (synthetic fox feces odor)
US	Unconditioned Stimulus
VGSC	Voltage-gated sodium channel
WAG/Rij	Wistar Albino Glaxo (rats) from Rijswijk
WT	Wildtype

CHAPTER 1: STRESS AND EPILEPSY: MULTIPLE MODELS, MULTIPLE OUTCOMES

Adapted from:

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1.1 Abstract

Human studies show a link between stress and epilepsy, with stress causing an increase in seizure frequency and severity in patients with epilepsy. Many different animal model systems have been used in order to better understand this connection and the possible mechanisms involved. This chapter highlights the results of such studies relating stress and seizure susceptibility, with a focus on the hypothalamic-pituitary-adrenal (HPA) axis and its relationship to seizure generation. The effects of HPA axis mediators, acute stress, chronic stress, and early life stress on the seizure phenotype are summarized. Results suggest that stress has both anti-convulsive and pro-convulsive properties, depending on the animal strain and the stress/seizure-induction paradigm used. Attempts to interpret the stress-epilepsy literature must take these variables into account. The growing availability of genetically modified mice that carry either human epilepsy mutations or mutations in stress-pathway genes now provide the opportunity to examine the relationship between stress and epilepsy more directly. The goal of this dissertation is to examine the effect of two voltage-gated sodium channel genes, *Scn8a* and *Scn1a*, on the stress response, the seizure response to stress, adult outcomes following altered early life experience, and behavior.

1.2 Introduction

When faced with a stressful situation, the body's physiological response has evolved to prepare an individual to assess the situation and act accordingly; hence, faster, more instinctual mechanisms regulated by the amygdala, hippocampus, and striatum take over, superseding the more logical and analytical functions of the frontal cortex (Arnsten 1998). The physiological stress response is often divided into two separate yet linked systems acting in a coordinated temporal manner. The rapid response to a stressor involves the sympathetic-adrenomedullary (SAM) system, which results in activation of the sympathetic nervous system, increased systemic levels of norepinephrine and epinephrine, and elevated levels of norepinephrine in the brain. The slower, longer-lasting response to a stressor involves the hypothalamic-pituitary-adrenal (HPA) axis (**Fig. 1.1**) and begins with the paraventricular nucleus (PVN) of the hypothalamus releasing corticotropin-releasing hormone (CRH), which then stimulates the pituitary to release adrenocorticotropic hormone (ACTH) into the bloodstream. ACTH stimulates release of glucocorticoids (cortisol in humans and corticosterone in rodents) from the adrenal cortex. Glucocorticoids mobilize peripheral energy stores, dampen the immune response, and act as mediators in negative feedback control of the HPA axis (De Kloet *et al.* 2005). The HPA axis and SAM system work in conjunction with one another to coordinate adaptive responses to a stressor. Although these responses are important for survival, they have the potential to become maladaptive under chronic activation, such as in modern society, where stressful situations have become the norm.

Epilepsy is a common disorder characterized by periods of abnormal neuronal excitability resulting in recurrent seizures. The paroxysmal nature of epilepsy suggests the action of endogenous or exogenous precipitating factors. A better understanding of these triggers could lead to better seizure control, and as such, these factors have been avidly sought for over a century (McQuarrie 1929). Most clinical studies to date have been based on patient interviews and subjective recall of seizure-precipitating events. Stress, and emotional stress in particular, is ranked consistently as the most common seizure trigger in patient perception studies, independent of the sub-type of epilepsy reported (Mattson 1991; Frucht *et al.* 2000; Haut *et al.* 2003; da Silva Sousa *et al.* 2005; Nakken *et al.* 2005; Sperling *et al.* 2008). In response to these subjective studies, some have argued that stress results in sleep deprivation and medication non-compliance, two seizure-precipitating factors that have been more tangibly linked to greater seizure frequency. However, there is also a connection between stress and seizures independent of any relationship with

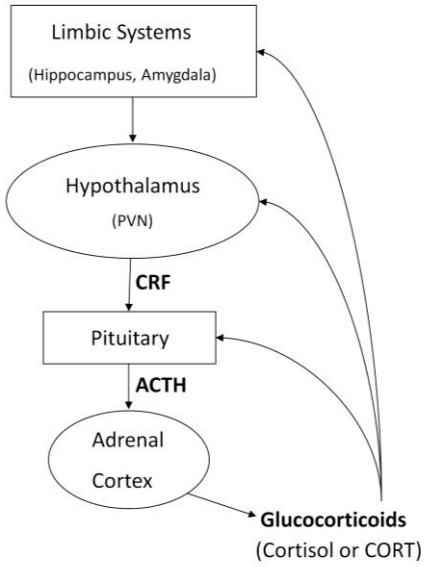

Figure 1.1 Hypothalamic-Pituitary-Adrenal (HPA) Axis. In response to a stressor, neurons in the paraventricular nucleus (PVN) of the hypothalamus release corticotropin-releasing hormone (CRH), which in turn stimulates adrenocorticotropic hormone (ACTH) release from the pituitary. ACTH stimulates the release of glucocorticoids (cortisol in humans and corticosterone in rodents) from the adrenal cortex. Glucocorticoids modulate the system by providing feedback at multiple levels via glucocorticoid receptors (GRs) and mineralocorticoid receptors (MRs).

sleep or medication non-compliance (Haut *et al.* 2007). Furthermore, patients with refractory epilepsy tend to report triggering factors, such as stress, more often than patients whose seizures are well controlled with medication, underscoring the potential therapeutic benefits of a better understanding of the relationship between stress and epilepsy (Sperling *et al.* 2008). Finally, in a study that did not rely on patient reporting, audio and video recordings designed to elicit empathetically stressful responses were sufficient to induce spontaneous seizures in epileptic patients (Feldman and Paul 1976). Taken together, these findings suggest that the physiological stress response is sufficient to provoke seizures in people with epilepsy.

This introduction will summarize human and animal studies that have examined the link between stress and epilepsy, highlighting the many different model systems used and the diversity of outcomes generated. Finally, we will introduce two genetic mouse models of epilepsy that will be used to further characterize the relationship between stress and epilepsy.

1.3 Human Studies

Human studies investigating the link between stress and epilepsy have focused primarily on HPA axis function in epileptic patients. As would be expected, since a seizure is in itself a stressful event, ACTH and cortisol levels are increased following both complex partial and generalized tonic-clonic seizures (Takeshita *et al.* 1986; Rao *et al.* 1989; Pritchard 1991). Reduced inhibitory control of the HPA axis is also seen in patients with epilepsy, as manifested by a prolonged elevation of cortisol levels following a stressor and a sluggish return to baseline levels (Rao *et al.* 1989; Zobel *et al.* 2004).

Other HPA axis abnormalities in epilepsy patients include increased basal levels of ACTH and cortisol and decreased levels of allotetrahydrodeoxycorticosterone (THDOC), a stress-related neuropeptide (Gallagher *et al.* 1984; Gallagher 1987; Galimberti *et al.* 2005; Tuveri *et al.* 2008). To examine the relationship between HPA axis function and recovery from status epilepticus, Calabrese *et al.* (1993) collected blood from adult patients within twelve hours after the cessation of seizing. The outcome of these patients six to twelve days later was correlated with the degree of HPA axis responsiveness; patients who did poorly (death or severe neurologic or medical complications) had significantly elevated plasma cortisol levels following status, whereas moderately elevated levels were seen in patients with a good outcome (little or no neurologic deficit) (Calabrese *et al.* 1993). Together, these studies point to HPA axis dysfunction in patients with epilepsy, but does this necessarily solidify a link between stress and increased seizure frequency?

One caveat when dealing with self-report studies is the subjective perception of stress itself. Therefore, a few studies have attempted to examine the relationship between stress and the occurrence of seizures in populations affected by a common stressor. Neufeld *et al.* (1994) investigated the emotional stress of the 1991 Persian Gulf War on Israelis with epilepsy and found a weak link between stress and seizure frequency; however, this study lacked a control population. Swinkels *et al.* (1998) examined the effect of a controlled evacuation from a flooded area in The Netherlands on epilepsy patients, using non-evacuee patients as controls, and found a strong relationship between the stressful situation and the frequency of seizures in the evacuated patients. Similarly, Bosnjak *et al.* (2002) compared children with epilepsy during the 1991-1992 Croatian

war to children with epilepsy from non-war-affected areas, and also found a strong link between stress and seizure frequency. However, beyond these types of correlational studies, human data on the link between stress and epilepsy are limited. It is for this reason that research has relied heavily on animal models to better probe the mechanistic underpinnings of the stress response and its relationship to epilepsy.

1.4 Animal Studies

1.4.1 Seizure Susceptibility and the HPA axis

One widely used epilepsy model involves kindling of the dorsal hippocampus or the amygdala in rodents; fully kindled animals display generalized seizures in response to mild electrical stimulation. Hippocampal kindling of Sprague-Dawley rats resulted in hyperactivity of the HPA axis as characterized by increased basal ACTH levels, and both hippocampal and amygdala kindling resulted in larger ACTH and corticosterone (CORT) responses to an acute stressor (Szafarczyk *et al.* 1986). However, Karst et al. (1997) reported that hippocampal kindling of Wistar rats resulted in lower basal levels of CORT during the kindling process and a faster return to baseline CORT levels following acute stress, indicating enhanced negative feedback and an adaptive HPA axis response in these animals. As both the hippocampus and the amygdala are involved in HPA axis regulation (Jankord and Herman 2008), it is not surprising that kindling these areas results in altered HPA axis activity (**Fig. 1.1**).

In another seizure model, analysis of Wistar rats following pilocarpine-induced status epilepticus revealed a hyperactive HPA axis, as evidenced by elevated basal levels of CORT, reduced suppression of CORT by administration of a synthetic glucocorticoid (dexamethasone), and a hypersecretion of CORT in response to a CRH challenge (Mazarati *et al.* 2009). Interestingly, although most of the rats developed spontaneous seizures following status epilepticus, abnormal HPA axis function was observed even in post-status rats that did not develop spontaneous seizures, suggesting that the epileptic brain itself is sufficient to produce an abnormal stress response (Mazarati *et al.* 2009).

Genetically seizure-susceptible animal models are also a powerful tool for examining the relationship between the seizure-susceptible brain and the stress response. For example, the Mongolian gerbil displays seizures when exposed to novelty, itself a stressor. Both CRH expression and CRH-receptor expression are up-regulated in the hippocampus shortly after seizure-sensitive gerbils experience a seizure, but expression levels quickly return to baseline, which does not differ from seizure-resistant gerbils (An *et al.* 2003; Park *et al.* 2003). Another genetic model of seizure susceptibility is the epilepsy (EL) mouse, which experiences seizures in response to vestibular stimulation. Forcelli et al. (2007) recently showed that these mice have higher CRH levels in the paraventricular thalamus and a higher CORT response to an acute stressor when compared to control mice; however, basal CORT levels in EL mice are comparable to controls (Forcelli *et al.* 2007).

1.4.2 Effects of CRH on Seizures

Many animal studies have focused on the effects of stress-related mediators on the seizure response. One such mediator is the CRH peptide, which functions not only as a hypothalamic releasing factor but also as a modulator of limbic circuit excitation (Baram and Hatalski 1998). Consistent with this role, a number of studies have shown CRH to be a pro-convulsive molecule. Intracerebroventricular (i.c.v.) injections of CRH reliably produced spontaneous seizures in wild-type Sprague-Dawley rats (Ehlers *et al.* 1983; Weiss *et al.* 1986; Marrosu *et al.* 1988; Baram and Schultz 1991) and CRH-induced epileptiform activity begins in the amygdala, reinforcing the view that CRH contributes to increased excitation in limbic regions (Baram *et al.* 1992). Furthermore, when CRH is administered prior to amygdala kindling, the time to reach the fully kindled state is markedly reduced (Weiss *et al.* 1986). In light of these data, it is possible that seizures triggered by emotional stress are a result of activation of the limbic circuit in conjunction with stress-induced CRH-mediated hyperexcitability. Consistent with this hypothesis, Lewis rats, which have inherently reduced CRH expression, take longer to kindle (i.e., more electrical stimulations are required), supporting the pro-convulsive role of CRH (Weiss *et al.* 1993).

1.4.3 Effects of ACTH on Seizures

Historically, ACTH is one of the most studied stress response mediators. Studies of the effects of ACTH in wild-type animals have revealed a combination of pro-

convulsive and anti-convulsive responses (reviewed in Holmes 1991). The only study looking at the effects of ACTH in a genetically seizure-susceptible model showed that the hormone can be anti-convulsive in Mongolian gerbils, but only at higher and potentially toxic doses (Bajorek *et al.* 1984). Interestingly, ACTH is used in the treatment of infantile spasms, a rare epileptic disorder in infants (Gupta and Appleton 2005). Administration of exogenous ACTH is suspected to down-regulate CRH expression and reduce limbic excitability (Brunson *et al.* 2002). Nevertheless, ACTH has been ineffective at treating other types of childhood epilepsies (Gayatri *et al.* 2007).

1.4.4 Effects of CORT on Seizures

CORT has also been associated with both pro-convulsive and anti-convulsive properties. In kindling models, CORT manipulations are primarily pro-convulsive, but a

Table 1.1 Kindling Studies Involving Corticosterone Manipulations

Brain Region Kindled	Corticosterone Manipulation	Rat Strain	Result	Effect*	Reference(s)
Amygdala or Hippocampus	Adrenalectomy (post-kindling)	Wistar	No Effect	N/A	(McIntyre 1976); (McIntyre and Wann 1978)
Amygdala	Hypophysectomy (pre-kindling)	Sprague-Dawley	Initial ↓ in Kindling Rate	Anti	(Rose et al. 1979)
Amygdala	Cortisone (i.p., 10 mg/rat, post-stimulation)	Sprague-Dawley	↓ Kindling Rate	Anti	(Rose et al. 1979) (Rose and Bridger 1982)
Hippocampus	Adrenalectomy (pre-kindling)	Wistar	Transient ↓ in Seizure Severity	Anti	(Cottrell et al. 1984)
Amygdala	CORT Pellet (200 mg)	Lewis (↓ CRF)	↑ Kindling Rate	Pro	(Weiss et al. 1993)
Hippocampus	CORT Pellet (100 mg/day)	Wistar	↑ Kindling Rate	Pro	(Karst et al. 1999)
Amygdala	CORT in Drinking Water (4.5 mg/kg/day)	Wistar	↑ Kindling Rate	Pro	(Taher et al. 2005)

* Convulsive effect: Anti (anticonvulsive) and Pro (proconvulsive).

handful of studies have shown either anti-convulsive effects or no effects at all (**Table 1.1**). One of the studies showing anti-convulsive effects involved removal of the pituitary, which alters far more than the HPA axis (Rose *et al.* 1979), and two studies used cortisone, a CORT metabolite, instead of CORT (Rose and Bridger 1982). It should be noted that many experimental variables differed between the kindling studies shown in **Table 1.1**: CORT administration was acute or chronic, adrenalectomy was performed either before or after the kindling process, CORT was administered via different methods and at different dosages, and different strains of rats were used. In light of these variations, the contradictory results are unsurprising. In an example of the influence of natural CORT fluctuations, wild-type Fischer-344 rats show a diurnal variation in kindling rates; rats are kindled faster in the evenings when endogenous CORT levels are at their highest, providing evidence that independent of experimental manipulation, CORT can act as a pro-convulsant (Weiss *et al.* 1993).

Variable results have also been obtained from studies using chemiconvulsants. Kainic acid-induced seizures were attenuated by adrenalectomy and enhanced by dexamethasone treatment in Fischer-344 rats, suggesting a pro-convulsive role for CORT (Lee *et al.* 1989). On the other hand, adrenalectomy significantly reduced bicuculline-induced seizure thresholds in both long-sleep and short-sleep mice, suggesting that it is the absence of CORT that is pro-convulsive (Bowers *et al.* 1991). Although one study used rats and the other study used mice, a more important distinction is the type of chemiconvulsant used. Kainic acid is an excitatory amino acid that enhances excitation, while bicuculline is a $GABA_A$ receptor antagonist that reduces inhibition. The types of

mechanisms by which chemiconvulsants act to induce seizures can have a major effect on the outcome of stress-epilepsy studies, as we will soon detail in the acute stress section.

What about the effect of CORT in genetic models? One of the earliest studies showed that the injection of cortisone into photo-sensitive baboons resulted in increased seizure activity in a dose-dependent manner (Ehlers and Killam 1979). Notably, seizure activity in these baboons followed a circadian rhythm, with maximal seizure activity correlating with maximal urine cortisol levels (Ehlers and Killam 1979). Moreover, the WAG/Rij rats, a model of absence epilepsy, show a rapid albeit transient increase in the number of absence seizures in response to the administration of CORT (Schridde and van Luijtelaar 2004). In contrast, administration of CORT to absence seizure-prone DBA/2J mice decreased the number of spontaneous spike and wave discharges observed (Capasso *et al.* 1994).

CORT exerts its effects on the brain through action at glucocorticoid receptors (GRs) and mineralocorticoid receptors (MRs). Downstream actions of these receptors can have opposing effects on neuronal excitability, and thus the balance of these receptors is critical for maintaining baseline sensitivity to input, particularly in limbic regions (De Kloet *et al.* 1998). It is likely that different animal models as well as different seizure induction paradigms influence the relative balance and distribution of GRs and MRs throughout the brain. Such differences could contribute to the disparities seen in experiments linking CORT with seizure activity.

The preceding sections summarized animal studies in which the relationship between individual HPA axis mediators (CRH, ACTH, CORT) and seizures were examined. While providing important information, such studies do not take the full

physiological stress response into account. The following sections will focus on studies that have utilized actual stressors. Exposure to a stressor results in a complete physiological stress response, and although such a response is complex and multi-faceted, these studies provide further insight into the relationship between stress and epilepsy.

1.4.5 Acute Stress and Seizures

The relationship between acute stress and seizure generation likewise seems variable and appears to be influenced by the animal model and method of seizure induction. In early studies, the application of alumina cream to the cortex was used to elicit the generation of spontaneous seizures in Rhesus macaque monkeys. Stress and the anticipation of a stressful event were identified as key triggering factors for these spontaneous seizures (Kopeloff *et al.* 1954; Lockard 1980). Intriguingly, the severity of the acute stressor correlated with the severity of the resulting seizure (Kopeloff *et al.* 1954). In the decades since, the focus has shifted to rodent models of induced epilepsy. Although not the main focus of their study, Cain and Corcoran (1984) noted that the handling and restraint necessary to administer injections increased the number of opioid-induced seizures in Hooded rats. Following up on this observation, they also showed that exposing subjects to a threatening male further exacerbated seizure activity (Cain and Corcoran 1984). In these studies, acute stress seemed to increase seizure activity.

In contrast, Soubrie *et al.* (1980) found acute stress to be anti-convulsive: four different acute stress paradigms all increased the threshold for picrotoxin-induced seizures in Wistar rats. Since 1980, many studies have investigated the hypothesis that

Table 1.2 Acute Swim Stress Studies

Water Temp	Swim Duration (min)	Rodent/Strain	Seizure-Induction Paradigm[1]	Result	Effect[2]	Reference(s)
6ºC	3	Wistar rats	Picro, PTZ, Thio, Iso	↑ Thresholds	Anti	(Soubrie et al. 1980)
			Bemegride, Strychnine	No Effect	N/A	
6ºC, 22ºC	10	NIH Swiss mice	Electroconvulsive Shock	↓ Efficacy of Flurazepam	Pro	(Deutsch et al. 1990)
22ºC	3	NIH Swiss mice	PTZ	↑ Threshold	Anti	(De Lima and Rae 1991)
			Electroconvulsive Shock	More Severe Seizures	Pro	
27ºC	10, 20	Sprague-Dawley rats	PTZ	↑ Threshold	Anti	(Abel and Berman 1993)
18-19ºC	10	Wistar rats	Bicuculline	↑ Threshold	Anti	(Pericic and Bujas 1997)
18-19ºC	10	CBA mice	Bicuculline, PTZ, Picro	↑ Threshold	Anti	(Pericic et al. 2000)
			Strychnine, 4-AP, KA	No Effect	N/A	(Pericic et al. 2001)

[1] Picro, picrotoxin; PTZ, pentylenetetrazol; Thio, thiosemicarbazide; Iso, isoniazid; 4-AP, 4-aminopyridine; KA, kainic acid

[2] Convulsive effect: Anti (anti-convulsive); Pro (pro-convulsive)

acute swim stress is anti-convulsive, with varying results (**Table 1.2**). The emerging consensus is that swim stress is anti-convulsive against chemiconvulsants with GABA-related seizure induction mechanisms. In addition, colder water temperatures (Pericic *et al.* 2001) and longer swim durations (Abel and Berman 1993) further increased resistance to seizures induced with GABA-related chemiconvulsants. However, swim stress does not appear to raise thresholds when seizures are induced using mechanisms that are GABA-unrelated, and in fact it exacerbates the response to electrically induced seizures (Deutsch *et al.* 1990; De Lima and Rae 1991). Adding further variability, Pericic and Bujas (1997) showed that the anti-convulsive effects of swim stress were sex-dependent, with females receiving the most protection. This is not surprising, given the known sex differences in HPA axis function (reviewed in Kudielka and Kirschbaum 2005). Interestingly, repeated swim stress (18-19ºC; 10 min) over five days in CBA mice

returned thresholds to picrotoxin-induced seizures to the levels seen in unstressed controls, indicating that the anti-convulsive properties of acute stress are indeed acute and not maintained following repeated stress exposure (Pericic *et al.* 2001).

Other studies have brought different insights into the effects of acute stress on seizure susceptibility. Rae *et al.* (1990) showed that conspecific aggression stress in NIH Swiss mice reduced thresholds to both PTZ- and pilocarpine-induced seizures and increased lethality to maximal transcorneal electroshock-induced seizures. One unique aspect of this study was that acute stress was not anti-convulsant against PTZ, a chemiconvulsant with a GABA-related seizure induction mechanism. Notably, this study uses aggression stress, suggestive of a larger limbic involvement in seizure generation. Finally, one study using Sprague-Dawley rats showed that foot shock stress reduced the latency to isoniazid-induced seizures and increased the number of animals that experienced a seizure in response to isoniazid, two pro-convulsive responses (Serra *et al.* 1991). These studies show that not only does the method of seizure induction matter, but the type of stressor may also influence the outcome of the experiment.

While the majority of studies examining the relationship between acute stress and seizure activity have used swim stress as the acute stressor, we have chosen to investigate the effects of an acute 20-minute restraint stress on seizure outcome in the experiments described in Chapters 2, 3, and 4. The main reason for this choice was that we wanted to measure the effects of the acute stress on spontaneous seizure activity using electroencephalogram (EEG) recordings, and the recording equipment utilized could not be exposed to water. While only a few studies have investigated the effect of restraint stress on seizure outcome, a recent study has shown that a 30-minute restraint stress can

reduce the number of stimulations required to fully kindle the amygdala (Jones *et al.* 2013). The experiments described within this thesis will not only allow us to characterize the interaction between epilepsy-causing mutations and stress, but it will also allow us to more fully characterize the effect of an acute 20-minute restraint stress on the seizure response in wildtype (WT) animals.

1.4.6 Chronic Stress and Seizures

Although fewer studies have looked at the effects of chronic stress on seizure susceptibility, the results have been more consistent. Mongolian gerbils subjected to a test regimen of 15-45 weekly novelty stress stimulations yielded two interesting findings. First, four groups of gerbils emerged that differed based on their seizure susceptibility, and unlike acute stress studies, no seizure-resistant gerbils were identified (Scotti *et al.* 1998). This model is likely polygenic, and the identification of four distinct subgroups suggests that the underlying neural circuitry is influenced by genetic factors that are important for shaping the seizure phenotype in response to chronic stress. A second finding of this study was that, over the course of the prolonged test regimen, seizures in response to weekly stimulations became progressively more severe, suggesting that chronic stress is not only pro-convulsive, but that it also influences the resulting seizure phenotype (Scotti *et al.* 1998).

Rodents are social animals, and chronic isolation produces physiological effects indicative of chronic stress (Van Loo *et al.* 2003). Two studies have used long-term social isolation as a model of mild chronic stress. In one study, social isolation over a

seven-week period increased the susceptibility of ddY mice to picrotoxin-induced seizures (Matsumoto *et al.* 2003). Interestingly, and reminiscent of the acute stress studies, these mice did not display any change in susceptibility to strychnine or kainic acid, two GABA-unrelated chemiconvulsants (Matsumoto *et al.* 2003). In contrast, Chadda and Devaud (2004) showed that individually housing Sprague-Dawley rats reduced thresholds of both sexes to bicuculline-induced, but not PTZ-induced seizures. In another example of sex differences in the stress response, an additional acute stressor (30-minute restraint stress) administered after 10 days of social isolation reduced bicuculline-induced seizure thresholds in individually housed males but, intriguingly, only in group-housed females (Chadda and Devaud 2004). In Chapter 4, we investigate the effect of chronic stress on spontaneous seizure generation in one of the genetic mouse models of epilepsy.

1.4.7 Early Life Stress and Seizures

Early life stress in both rodents and primates results in adults with altered stress responses (reviewed in Sanchez *et al.* 2001). In a study examining the effects of progesterone metabolites on seizure susceptibility, allopregnanolone was found to be anti-convulsive against kainic acid-induced seizures in Long Evans rats; however, prenatal stress attenuated this effect (Frye and Bayon 1999), suggesting that the early life experience had a pro-convulsive effect that was sufficient to overcome the anti-convulsive properties of allopregnanolone. Subsequent studies using Sprague-Dawley rats also showed early life stress to be pro-convulsive. Edwards *et al.* (2002)

demonstrated that late gestational stress increased the kindling rate in young rats (P13) of both sexes, but increased the kindling rate only in adult male rats. Huang *et al.* (2002) isolated rat pups from their dam for one hour per day from P2 to P9, and seizures were induced using PTZ the following day. Pups that experienced maternal separation exhibited seizures of a longer duration (Huang *et al.* 2002). Lai *et al.* (2006) also used a similar combination of maternal separation and seizure induction. Rat pups were isolated from their dam for one hour per day from P2 to P9 and exposed to pilocarpine-induced status epilepticus on P10. Maternal separation alone or pilocarpine-induced status epilepticus alone did not lower seizure threshold in adult animals, but the combination of both maternal separation and status on P10 resulted in adults with a significant reduction in thresholds to PTZ-induced seizures (Lai *et al.* 2006). In addition, using Wistar rats, Salzberg *et al.* (2007) showed that maternal separation from P2 to P14 resulted in a faster amygdala kindling rate in adult females, but not adult males. Opposite sex effects were observed in two of these studies: early life stress reduced the kindling rate only in adult males in one study (Edwards *et al.* 2002) and only in adult females in the other study (Salzberg *et al.* 2007). But these studies differed in three important ways and offer an example of how the model used affects the outcome: Edwards *et al.* (2002) used Sprague-Dawley rats, prenatal stress, and hippocampal kindling, whereas Salzberg *et al.* (2007) used Wistar rats, postnatal stress, and amygdala kindling. In either case, early life stress proved to be pro-convulsive in at least one sex.

Recently, the influence of the early life environment on seizure activity was investigated in the epilepsy (EL) mouse. Leussis and Heinrichs (2009) cross-fostered EL pups to CD-1 dams because CD-1 dams exhibit a higher quality of maternal care than EL

dams. EL pups raised by CD-1 dams experienced delayed seizure onset and reduced seizure frequency, suggesting that early life environment can play an important role in shaping the adult seizure phenotype (Leussis and Heinrichs 2009). Interestingly, EL pups raised in a biparental environment with both the EL dam and sire attending the pups, received more parental attention, yet showed lower body weights and an earlier development of seizures than EL pups raised by only the El dam (Orefice and Heinrichs 2008). Although the EL mouse model has not been used to test the effects of early life stress per se, these two studies demonstrate that the early life environment can interact with genetic predisposition to shape the future seizure phenotype. In Chapter 3, we will use a model of altered early life experience to further characterize the relationship between events experienced early in life and the resulting adult seizure phenotype in a mouse model of epilepsy.

1.5 Possible Mechanisms

Given the putative anti-convulsive and pro-convulsive effects of stress, we would like to highlight a few key mechanisms and point out other more extensive reviews on this topic. Much attention has been focused on the mechanisms behind the anti-convulsive effects of acute stress. The key players seem to be neuroactive steroids like THDOC and allopregnanolone, which act as positive modulators of the $GABA_A$ receptor, increasing inhibitory tone and reducing seizure susceptibility (reviewed in Reddy 2006; Morrow 2007). Barbaccia *et al.* (2001) reviewed studies showing how acute stress increases the levels of anti-convulsant neuroactive steroids, while chronic stress reduces

their levels. Belelli *et al.* (2006), on the other hand, offers a more mechanistic review, focusing on how neuroactive steroids can selectively modulate GABA activity, depending on subunit composition, steroid metabolism, phosphorylation, and other regulatory mechanisms. In light of the sex differences like the ones described above, review articles have also included the effects of the sex hormones on brain excitability (Beyenburg *et al.* 2001) as well as the effects of stress and seizures on sex hormones and seizure activity resulting from altered hormonal levels (Rhodes *et al.* 2004).

As we have seen, stress can also have pro-convulsive effects. Joels (2009) offers an extensive review of the cellular effects of stress mediators (e.g., CRH, CORT) in the rodent hippocampus, positing mechanisms by which stress can switch from being anti-convulsive to pro-convulsive. Baram and Hatalski (1998) propose that the excitatory properties of CRH can lead to the development of epileptogenesis in the young brain, which is already inherently hyperexcitable. Finally, the stress response entails not only activation of the HPA axis, but the SAM system as well. In particular, norepinephrine is known to affect seizure activity in ways both pro- and anti-convulsive (reviewed in Giorgi *et al.* 2004). Furthermore, norepinephrine and CRH are intrinsically linked, as neurons from the locus coeruleus project to the PVN, and neurons from the PVN project back to the locus coeruleus, making true separation of the HPA axis and SAM system difficult, at best (reviewed in Koob 1999).

1.6 Multiple Models; Multiple Outcomes

This introduction shows that the link between stress and epilepsy has been explored in a range of animal models. Within rodent models, many different strains of rats and mice have been used. Notably, the majority of these studies were conducted in wild-type animals with normal brain and HPA axis development that was only perturbed when the investigator induced an epileptic state by electrical or chemical means. Although such studies may offer important insights into the relationship between stress and symptomatic epilepsies acquired during adulthood, they may not fully reflect the relationship between stress and the idiopathic epilepsies, which often manifest in early childhood and have a strong genetic component. The epileptic brain resulting from a genetic mutation likely develops differently, leading to altered network connectivity early in development. Some genetically seizure-susceptible models, such as the EL mouse, have been used to investigate the link between stress and epilepsy; however, these models are likely polygenic. Since the stress response is multi-faceted and complex, monogenic models would provide a simpler system for teasing out key mechanisms linking stress and epilepsy.

To illustrate this concept, consider patients with mutations in the voltage-gated sodium channel gene *SCN1A.* In these patients, seizures usually develop early in childhood, and the current hypothesis is that these mutations attenuate sodium currents and reduce the excitability of inhibitory neurons, thereby decreasing overall network inhibition (Yu *et al.* 2006; Ragsdale 2008; Gambardella and Marini 2009; Tang *et al.* 2009; Martin *et al.* 2010). As the HPA axis is controlled by a combination of inhibitory and excitatory signals from limbic regions (Herman *et al.* 2005), a disruption in the

balance between inhibition and excitation could alter the functioning of the HPA axis, and thus the response to a stressor. The growing availability of mouse models with human epilepsy mutations, as well as mouse models with mutations in stress-pathway genes, will provide new opportunities to investigate the contribution of single genes to both seizure susceptibility and the stress response.

1.7 Genetic Mouse Models of Epilepsy

The experiments discussed in this dissertation aim to examine the relationship between stress and epilepsy more directly by using genetic mouse models of epilepsy. Both models used in the experiments described herein are voltage-gated sodium channel (VGSC) mutants. The mammalian genome contains four VGSC α-subunit genes that are highly expressed in the central nervous system. These genes are *SCN1A*, *SCN2A*, *SCN3A*, and *SCN8A*, which code for the transmembrane proteins $Na_v1.1$, $Na_v1.2$, $Na_v1.3$, and $Na_v1.6$, respectively. VGSCs facilitate the initial and propagation of transient depolarizing currents and electrical signaling between cells in the central nervous system.

The first mouse model used in these experiments is the *Scn8a*^{med} mutant which carries a null mutation in *Scn8a* (encoding $Na_v1.6$). $Na_v1.6$ is the most widely expressed VGSC in the central nervous system (Schaller *et al.* 1995; Krzemien *et al.* 2000) and plays a major role in the transmission of subthreshold currents, namely persistent current and resurgent current (Raman *et al.* 1997). The electrophysiological properties of $Na_v1.6$ make it especially suited for the sustained repetitive firing of neurons (Van Wart and Matthews 2006), a key feature of many neuronal circuits. The *Scn8a*^{med} mutation arose

spontaneously in a mouse colony in 1958 (*http://jaxmice.jax.org/strain/003798.html*). Homozygous *Scn8a*$^{med/med}$ mutants display muscle weakness, progressive paralysis, and premature death (Burgess *et al.* 1995). Heterozygous *Scn8a*$^{med/+}$ mutants, on the other hand, exhibit normal gross motor behavior and live a normal lifespan. Our lab has shown that the *Scn8a*$^{med/+}$ mutants have a dual seizure phenotype: they display spontaneous spike-wave discharges (SWDs) characteristic of absence epilepsy (Papale *et al.* 2009), but they also have a greater resistance to chemically induced seizures (Martin *et al.* 2007). The *Scn8a*$^{med/+}$ mutants, therefore, have provided us with an opportunity to investigate the consequences of altered VGSC function on the stress response and behavior, as well as allowing us to explore the effect of stress on both spontaneous and induced seizures. In Chapter 2, we investigate the relationship between acute stress and the *Scn8a*$^{med/+}$ mutation, while in Chapter 3 we investigate the relationship between altered early life experience and the *Scn8a*$^{med/+}$ mutation.

The second mouse model used in these experiments is the *Scn1a*^{RH} mutant. These mutants express the human *SCN1A* (encoding $Na_v1.1$) missense mutation (R1648H). In the central nervous system, $Na_v1.1$ is localized predominantly to inhibitory PV-interneurons (Dutton *et al.* 2012), and mutations in *Scn1a* result in a reduction of the excitability of these inhibitory interneurons thereby decreasing overall network inhibition and increasing neural excitability (Yu *et al.* 2006; Ragsdale 2008; Tang *et al.* 2009; Martin *et al.* 2010). As a consequence of this disruption of inhibition, *Scn1a* mutants display spontaneous generalized seizures. The R1648H mutation was identified in a family with genetic epilepsy with febrile seizures plus (GEFS+) (Escayg *et al.* 2000), a disease characterized by febrile seizures that persist beyond six years of age and the

development of afebrile generalized or partial seizures during adulthood (Scheffer *et al.* 2005). The *Scn1a*^{RH} mutant was created by knocking-in the human mutation into the orthologous mouse gene (Martin *et al.* 2010). Homozygous *Scn1a*$^{RH/RH}$ mutants exhibit frequent spontaneous generalized seizures and premature lethality, while heterozygous *Scn1a*$^{RH/+}$ mutants exhibit infrequent spontaneous seizures and lower thresholds to flurothyl- and hyperthermia-induced seizures (Martin *et al.* 2010). Interestingly, within GEFS+ families, affected members with the same mutation display a wide range of epilepsy subtypes and severities, suggesting other genetic, developmental, and/or environmental factors may play a role in modulating seizure activity (Scheffer and Berkovic 1997). The *Scn1a*$^{RH/+}$ mutant, therefore, has provided us with an opportunity to investigate the consequences of altered VGSC function on the stress response and behavior, as well as allowing us to explore the contribution of stress to the varying phenotypes seen in GEFS+ families. These experiments are detailed in Chapter 4.

Figure 1.2 shows a schematic of a voltage-gated sodium channel showing the locations of the *Scn8a*^{med} and *Scn1a*^{RH} mutations. **Table 1.3** provides a side-by-side look at these two models used in the experiments described within this dissertation.

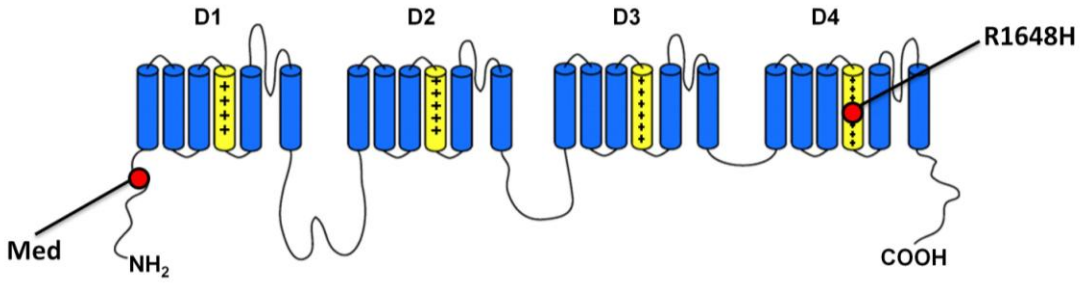

Figure 1.2 A schematic of the α subunit of a voltage-gated sodium channel. This subunit consists of four homologous domains (D1-D4), each containing six transmembrane segments. The voltage-sensing S4 segment is shown in yellow and has multiple, positively charged amino acids (+). The *Scn8a*^{med} mutation is a null mutation that results in a truncated protein near the N-terminus. The *Scn1a*^{RH} mutation is a missense mutation located in the voltage-sensing S4 segment of the fourth domain (D4).

Table 1.3 Mouse Models of Genetic Epilepsy Used in Described Experiments

	Scn8a^{med}	*Scn1a*^{RH}
Type of Mutation	Null	Missense; Loss of Function
Location of Mutation in Protein	N-Terminus	Voltage Sensor of S4 Segment
Types of Neurons Affected	Excitatory Pyramidal Neurons & Inhibitory Interneurons	Inhibitory Interneurons
Susceptibility to Convulsive Seizures	Increased threshold to induced seizures	Decreased threshold to induced seizures
Associated Seizure Disorder	Absence Seizures (SWDs)	GEFS+
Background of Mouse Model	C3HeB/FeJ	C57BL/6J

1.8 Conclusion

Stress has long been suspected of playing a role in increasing seizure frequency in epilepsy patients. More recently, studies of patients with epilepsy have provided scientific evidence for this link and shown altered stress system responsiveness in patients with epilepsy. In an attempt to better understand the interplay between stress and epilepsy, animal models have been helpful, but the results are often contradictory. The majority of animal studies have been conducted in wild-type rodents using a variety of stress and/or seizure induction paradigms. Though stress mediators like CRH are consistently shown to be pro-convulsive, other mediators, such as ACTH, corticosterone, and norepinephrine, show both pro-convulsive and anti-convulsive properties. Acute stress is also known to both increase and decrease seizure susceptibility, depending on the seizure induction method and the type of acute stressor used. Chronic stress and early life stress, on the other hand, consistently result in pro-convulsive outcomes. Efforts to identify precise mechanisms linking stress and seizures are confounded by the use of many models, yielding many outcomes. Mouse models carrying single mutations, such as

the VGSC mutants, may be preferable, offering less complex systems in which to examine the relationship between stress and epilepsy.

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CHAPTER 2: *SCN8A* VOLTAGE-GATED SODIUM CHANNEL MUTATION ALTERS SEIZURE AND ANXIETY RESPONSES TO ACUTE STRESS

Adapted from:

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2.1 Abstract

Stress is known to trigger seizures in patients with epilepsy, highlighting the physiological stress response as a possible therapeutic target for epilepsy treatment. Nevertheless, little is currently known about how a genetic predisposition to epilepsy interacts with the stress response to influence seizure outcome. To address this question, we examined the effect of acute stress on seizure outcome in mice with mutations in the voltage-gated sodium channel (VGSC) gene *Scn8a*. *Scn8a* mutants display spontaneous spike-wave discharges (SWDs) characteristic of absence epilepsy. We saw that the baseline frequency of SWDs in *Scn8a* mutants correlates closely with the diurnal activity of the hypothalamic-pituitary-adrenal (HPA) axis, with a peak in seizure activity occurring at around the same time as the peak in corticosterone (5-7 pm). A 20-minute acute restraint stress administered in the morning increases the frequency of spontaneous SWDs immediately following the stressor. Seizure frequency then returns to baseline levels within three hours after stressor exposure, but the subsequent evening peak in seizure frequency is delayed and broadened, changes that persist into the next evening and are accompanied by long-lasting changes in HPA axis activity. *Scn8a* mutants also show increased anxiety-like behavior in mildly stressful situations. A 20-minute acute restraint stress can also increase the severity and duration of chemically induced seizures in *Scn8a* mutants, changes that differ from wild-type littermates. Overall, our data show that a voltage-gated sodium channel mutation can alter the behavioral response to stress and can interact with the stress response to alter seizure outcome.

2.2 Introduction

Seizures are paroxysmal events, suggesting the presence of endogenous or environmental triggering factors. Epilepsy patients self-report stress as being the most common trigger for seizure onset (Spatt *et al.* 1998; Haut *et al.* 2003; Nakken *et al.* 2005; Haut *et al.* 2007), an assertion further supported by controlled studies that establish a relationship between stress and increased seizure frequency (Feldman and Paul 1976; Swinkels *et al.* 1998). In at least one study, the exacerbating effects of stress on seizure incidence was especially apparent in patients with typical absence seizures (Bosnjak *et al.* 2002), a subtype of epilepsy characterized by a brief loss of awareness associated with bursts of generalized synchronous spike-wave discharges (SWDs). Although stress and seizures are consistently linked, the physiological mechanisms that connect stress and seizure incidence or severity are not fully understood.

The acute stress response involves activation of the hypothalamic-pituitary-adrenal (HPA) axis, which results in the sequential release of corticotropin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH), and corticosterone (CORT). Receptors for both CRH and CORT can be found in numerous brain areas, such as the hippocampus and amygdala, suggesting these stress mediators can directly influence neuronal activity and excitability in limbic regions that are also important for seizure activity (Joels and Baram 2009). In addition to stress, anxiety levels predict changes in seizure frequency (Thapar *et al.* 2009), and hyperemotionality may underlie the majority of behavioral problems in epilepsy (Frucht *et al.* 2000). Furthermore, patients with epilepsy have higher incidences of depression, anxiety, and even psychosis compared to the general population (Garcia-Morales *et al.* 2008). HPA axis function modulates

activity in limbic regions, and dysfunction of the HPA axis is implicated in a number of affective disorders. Therefore, it has been hypothesized that the HPA axis could mediate both stress-induced seizures and behavioral alterations in seizure models (Heinrichs 2010). Given the bidirectional relationship between hyperemotionality, stress, and epilepsy, a genetic mouse model of epilepsy would provide a unique opportunity for investigating the stress response and affective-like behaviors and may provide important insight into the pathophysiological effects of stress on seizure incidence and severity.

A *de novo* gain-of-function point mutation in *SCN8A* was recently identified in a individual who exhibited generalized seizures and sudden unexplained death in epilepsy (SUDEP) (Veeramah *et al.* 2012) while other *SCN8A* mutations have been linked to epileptic encephalopathy and intellectual disability (O'Brien and Meisler 2013). The *SCN8A* gene encodes the α-subunit of the $Na_v1.6$ voltage-gated sodium channel (VGSC). VGSCs are transmembrane complexes that facilitate the initiation and propagation of transient depolarizing currents and electrical signaling between cells in the nervous system. $Na_v1.6$ is the most widely expressed VGSC in the central nervous system, including areas involved in the stress response such as the hippocampus and hypothalamus (Schaller *et al.* 1995; Krzemien *et al.* 2000). $Na_v1.6$ plays a major role in the transmission of subthreshold currents, namely persistent current and resurgent current (Raman *et al.* 1997), and the electrophysiological properties of $Na_v1.6$ make these channels especially suited for the sustained repetitive firing of neurons (Van Wart and Matthews 2006), a key feature in many neuronal circuits. In addition to its role in seizure activity, a two-bp deletion in *SCN8A* was found to co-segregate with motor and cognitive deficits in a small pedigree comprising six affected members (Trudeau *et al.* 2006).

Furthermore, findings from two association studies point to possible roles for *SCN8A* in emotional instability, suicide, and bipolar disorder (Wasserman *et al.* 2005; Wang *et al.* 2008).

Today there are several mouse models of *Scn8a* dysfunction. Null mutants, such as the *Scn8a*^{med} mouse, are the most severe, with homozygous *Scn8a*$^{med/med}$ mice showing muscle weakness, progressive paralysis, and premature death (Burgess *et al.* 1995). In contrast, heterozygous *Scn8a*$^{med/+}$ mice exhibit normal gross motor behavior and a normal lifespan. Interestingly, the *Scn8a*$^{med/+}$ mice have increased resistance to chemically induced seizures (Martin *et al.* 2007), but exhibit frequent SWDs characteristic of absence epilepsy (**Fig. 2.1**A) (Papale *et al.* 2009). Other *Scn8a* mutants, such as *Scn8a*$^{med\text{-}jo}$ and *Scn8a*^{8J}, also display spontaneous SWDs (Papale *et al.* 2009), and greater resistance to chemically induced seizures and hippocampal kindling have been seen in *Scn8a*$^{med\text{-}jo}$ and *Scn8a*^{tg} mutants, respectively (Martin *et al.* 2007; Blumenfeld *et al.* 2009). Increases in both anxiety-like and depressive-like behaviors have been reported in the *Scn8a*^{tg} mutant (McKinney *et al.* 2008), while our lab previously found only minor changes in anxiety-like behavior in the *Scn8a*$^{med\text{-}jo}$ mutant (Papale *et al.* 2010). *Scn8a* mutants, therefore, provide the opportunity to investigate the consequences of altered sodium channel function on the stress response and behavior, as well as the interaction between stress and spontaneous and induced seizures.

The goal of this study was to investigate the interaction between acute stress and epilepsy using the *Scn8a*$^{med/+}$ mouse to answer these four questions: (i) Does acute stress alter the frequency of spontaneous absence seizures? (ii) Does acute stress affect seizure

thresholds? (iii) Does sodium channel dysfunction affect HPA axis function? and (iv) Does sodium channel dysfunction alter anxiety-like or depressive-like behaviors?

2.3 Methods and Materials

Subjects

Scn8a$^{med/+}$ mice were purchased from The Jackson Laboratories (Bar Harbor, ME) and maintained on the C3HeB/FeJ background. Genotyping was performed on tail DNA using The Jackson Laboratory protocol. Male mice, 3-4 months old, were used for all experiments. Wild-type (WT) littermates were used as controls for all experiments. Mice were group-housed after weaning in ventilated cages under uniform conditions with a 12/12 h light/dark cycle with lights on at 7 am and lights off at 7 pm. Food and water were available *ad libitum*. All behavioral tests and all stressors were administered between the hours of 8 am and 11 am to minimize variation due to circadian factors and were performed in rooms with lighting of approximately 1,000 lux. All experiments were approved by the Emory University Institutional Animal Care and Use Committee (IACUC).

Acute stressor

Mice were placed into an adequately ventilated clear polypropylene restrainer (50-mL conical tubes measuring 9.7 cm in length with an internal diameter of 2.8 cm) for 20 min. Breathing was carefully observed to ensure the animals were not compressed.

EEG analysis of spontaneous seizure activity

Under isoflurane anesthesia, mice were surgically implanted with electroencephalogram (EEG) and electromyography (EMG) electrodes for seizure monitoring as previously described (Martin *et al.* 2007; Papale *et al.* 2009; Papale *et al.* 2010). Following 7 d of recovery, the animals were singly housed in Plexiglas boxes (20x20x30 cm^3), and a microconnector was attached to a series of bioelectric amplifiers (Stellate Harmonie software, Natus Medical Inc., California, USA) via a small counterbalanced commutator (Dragonfly Research, West Virginia, USA). Following 24 h of habituation, continuous EEG and EMG data were collected for a 48-h baseline period. The amplified signals were digitally acquired, collected, and processed by Stellate. Data were manually analyzed for the number and duration of spike-wave discharges (SWDs), the electroencephalographic feature of absence epilepsy. SWDs were defined by a rhythmic oscillation with a burst frequency of 7-9 Hz lasting at least 0.5 s, with an amplitude at least twice that of the background. To examine circadian variation, SWDs were grouped into 2-h intervals and averaged within each interval over day 1 and day 2 of the baseline recordings. Following the baseline recording, the seizure response to acute restraint stress was determined in a subset of animals by placing animals into modified restraint tubes (50-mL conical plastic tube with a 1.5x1.5 cm^2 hole cut out for EEG headcap placement) for 20 min. Continuous EEG and EMG data were collected for the 20-min restraint period, as well as for the 24-h period following restraint (post-restraint, PR). To examine the response to stress, SWDs were analyzed in 2-h intervals for the 24-h PR period. The first 3-h PR period was also analyzed by further sub-dividing SWD occurrence into 20-min bins. The total time the animals spent struggling while in the tube was determined by the presence of EMG

activity during the 20-min restraint period. A separate group of 8 *Scn8a*$^{med/+}$ mutants was used to determine how long post-stress changes in SWD activity persisted. These animals were implanted with EEG electrodes and were recorded for 48 h of baseline, followed by a 20-min restraint stress and 5 d PR.

Chemiconvulsant seizure induction

Mice were brought into the procedure room 2 h prior to the experiment for acclimation. Half of the mice were subjected to an acute 20-min restraint stress just prior to injection, while the other half served as unstressed controls. Mice were injected intraperitoneally (i.p.) with either 5 mg/kg picrotoxin (Sigma) or 30 mg/kg kainic acid (Sigma). Both drugs were dissolved in 0.9% saline, and all mice were injected at a volume of 10 mL/kg. Picrotoxin-injected mice were observed for 1 h, and kainic acid-injected mice were observed for 2 h post-injection. Mice were scored for seizure latencies and progression according to modified Racine scales (Racine 1972) described as follows. Picrotoxin is a noncompetitive $GABA_A$ receptor antagonist that induces seizures by reducing overall inhibition (Velisek 2006). Picrotoxin-induced seizure activity was scored based on the following criteria recommended for seizures induced by disruption of the GABAergic system (Veliskova 2006): 0) No behavioral response, 0.5) Abnormal behavior (freezing, staring, orientation problems), 1) Isolated myoclonic jerks, 2) Atypical clonic seizure, 3) Fully developed bilateral forelimb clonus, 3.5) Forelimb clonus with a tonic component and twist of the body, 4) Generalized tonic-clonic seizure (GTCS) with a suppressed tonic phase, and 5) Fully developed GTCS with hindlimb extension. Kainic acid is a specific agonist for the glutamatergic kainate receptor and induces seizures by increasing excitability in limbic regions (Velisek 2006). Kainic acid-induced seizure activity was

scored based on the following recommended criteria (Veliskova 2006): 0) No behavioral response, 1) Head nodding and/or staring with mouth clonus, 2) Automatisms (excessive washing, sniffing, rearing, scratching, circling, orientation problems), 3) Unilateral forelimb clonus, 4) Bilateral forelimb clonus, 5) Bilateral forelimb clonus with rearing and falling, 6) GTCS. If at the end of the observation period a mouse had not progressed through all of the seizure stages as described above, they were scored the maximum amount of time (60-min for picrotoxin; 120-min for kainic acid) for each stage they had not reached.

Diurnal corticosterone profile

Mice were single-housed a week prior to the experiment. Blood was collected at 7 am and 7 pm in order to coincide with the expected nadir and peak of circulating corticosterone (CORT) levels. In a separate group of animals, the effect of a morning stressor on evening CORT levels was measured. Animals were single-housed for one week prior to the experiment. During the first experimental week, blood was collected at 7 pm to establish baseline 7 pm CORT levels. A week later, blood was drawn from each animal between 8:30-8:40 am for a basal measurement prior to a 20-min restraint stress, and then again at 7 pm for a post-stress evening CORT measurement. For all CORT measurements, blood was obtained within 1 min of cage disturbance. Blood was collected from the facial vein into Microvette CB 300 Z tubes (Starstedt) and then allowed to clot for 1 h at room temperature. The serum was then separated by centrifugation at 5600 rpm for 15 min at 4°C and stored at -80°C until analysis. Serum CORT levels for the diurnal blood draws were assayed using a commercial radioimmunoassay (RIA) kit (MP Biomedicals) according to the manufacturer's instructions.

Corticosterone response to acute stress

Mice were subjected to a 20-min acute restraint stress. At six different time points (once before the stressor (baseline) and at 5, 30, 45, 60, and 120 min following the 20-min restraint stress) one group of animals was anesthetized with isoflurane, decapitated, and their trunk blood was collected into Microvette CB 300 Z tubes (Starstedt). The blood was allowed to clot for 1 h at room temperature, and then separated by centrifugation at 5600 rpm for 15 min at 4°C and stored at -80°C until analysis. Measurement of the serum CORT levels in response to an acute stressor was conducted by the Yerkes National Primate Research Center Biomarkers Core Lab (Atlanta, GA) using a commercially prepared kit (Siemens).

Predator odor stress

Predator odor exposure occurred in an enclosed transparent PVC box (30x30x30 cm^3) fitted with a small shelf to hold a single piece of filter paper. Synthetic fox feces odor, 2,5-dihydro-2,4,5-trimethylthiazoline (TMT, Contech, Victoria, Canada), was diluted 1:10 in ethanol. The mice were single-housed for one week prior to testing. Animals were brought into a procedural holding room 2 h prior for acclimation. Animals were transported one at a time to the testing room. On the first day, animals were placed into the control box, and 35 µL of vehicle (ethanol) was placed on a small piece of filter paper. The filter paper was placed on the ledge inside the box, the box was closed, and the animal was observed and videotaped for 15 min. Forty-eight h later, the animal was placed into the odor box, and 35 µL of diluted TMT was placed on the filter paper. The animal was again observed and videotaped for 15 min. After an additional 48-h period,

the animal was returned to the odor box, and 35 µL of vehicle was again placed on the filter paper to determine if repeated exposure to the same environment affected freezing levels. The animal was observed and videotaped a third time. Videotapes were later scored by an observer blind to genotype and condition. Mice were scored for total freezing time (defined as the lack of movement, except for respiration and heart beat) and number of rearing occurrences.

Novelty stress

The mice were single-housed for one week prior to testing. Animals were brought into the procedure room 2 h prior to testing for acclimation. To measure response to novelty, animals were placed into a new standard mouse cage (30x17 cm^2) containing only corncob bedding. Animals were observed and videotaped for 15 min. Videotapes were later scored by an observer blind to genotype. Mice were scored for grooming and exploratory behaviors (rearing and stretch-attends) by counting the number of individual occurrences of these behaviors.

Forced swim stress

The forced swim apparatus is composed of a 4-L glass beaker (24 cm height x 18 cm diameter) filled three quarters full with water. Water temperature was maintained at 25 ± 1.5°C. Animals were brought into the procedure room 2 h prior to testing for acclimation. Mice were placed individually into the cylinder for 10 min and videotaped. Videotapes were later scored by an observer blind to genotype. Latency to first immobility and the total durations of immobility and struggling during the test period were scored. A mouse was judged to be immobile when making only those movements necessary to keep its

head above water. Struggling was defined as vigorous movements with forepaws breaking the surface of the water.

Statistics

Data are reported as mean ± standard error of the mean (SEM). A two-tailed t-test was used to compare WT and *Scn8a*$^{med/+}$ mice for the total numbers of SWD, average durations of SWD, total time spent in SWD activity, absolute change in SWD frequency following restraint stress, average latency to first SWD following stress, baseline CORT prior to stress, amount of struggle, amount of grooming, number of stretch-attends, and number of rears. Parametric data sets with 2 or more groups/factors to be compared were analyzed using two-way analysis of variance (ANOVA). In experiments where all of the groups/conditions contained different sets of animals and there was only one measurement per animal (CORT response to acute stress; induced seizure thresholds) a two-way ANOVA was used. In experiments where there were multiple data values from a single animal (multiple time points in the 24-h rhythm of SWD; 7 am and 7 pm in the diurnal CORT profile; baseline SWD and SWD response to acute stress over multiple time points; predator odor response over three test days) a two-way repeated measures ANOVA (rANOVA) was used. Following the ANOVA analyses, the Tukey pairwise comparison test was used to further distinguish among groups. Nonparametric data (Racine scores) were analyzed using the Mann-Whitney Rank Sum test. Dichotomous data (presence/absence of GTCS) were analyzed using Fisher's Exact test. All results were considered statistically significant if $p<0.05$.

2.4 Results

2.4.1 *Scn8a*$^{med/+}$ mice have frequent spontaneous SWDs

The *Scn8a*^{med} line is maintained on a C3HeB/FeJ background, and wild-type (WT) animals from this background strain exhibit low levels of spontaneous spike-wave discharges (SWDs) (Frankel *et al.* 2005). Nevertheless, *Scn8a*$^{med/+}$ mice have significantly more SWDs in a 24-h period than the WT littermates as we have previously reported (Papale *et al.* 2009), and the average duration of the SWDs exhibited by the mutants is longer than that of the WT littermates (**Fig. 2.1**B,C). Consequently, *Scn8a*$^{med/+}$ mice spend a significantly longer time engaged in SWD activity over a 24-h period than the WT littermates (**Fig. 2.1**D).

2.4.2 Acute stress increases SWD frequency in *Scn8a*$^{med/+}$ mice

Scn8a$^{med/+}$ mutants and WT littermates were restrained for 20 min while simultaneous EEG recordings were obtained. A statistically significant increase in SWD activity in the 2-h period immediately following the acute stress exposure was seen only in the mutants (**Fig. 2.2**A). A closer look at the absolute change in SWD activity for each animal (ΔSWD Frequency = $SWD_{post\text{-}restraint} - SWD_{baseline}$) over this 2-h period shows that the *Scn8a*$^{med/+}$ mice had a significantly larger change than the WT littermates (**Fig. 2.2**B). To better characterize the temporal effect of stress on SWD activity in the mutants, the 3-h period following the restraint stress was subdivided into 20-min bins. Following an

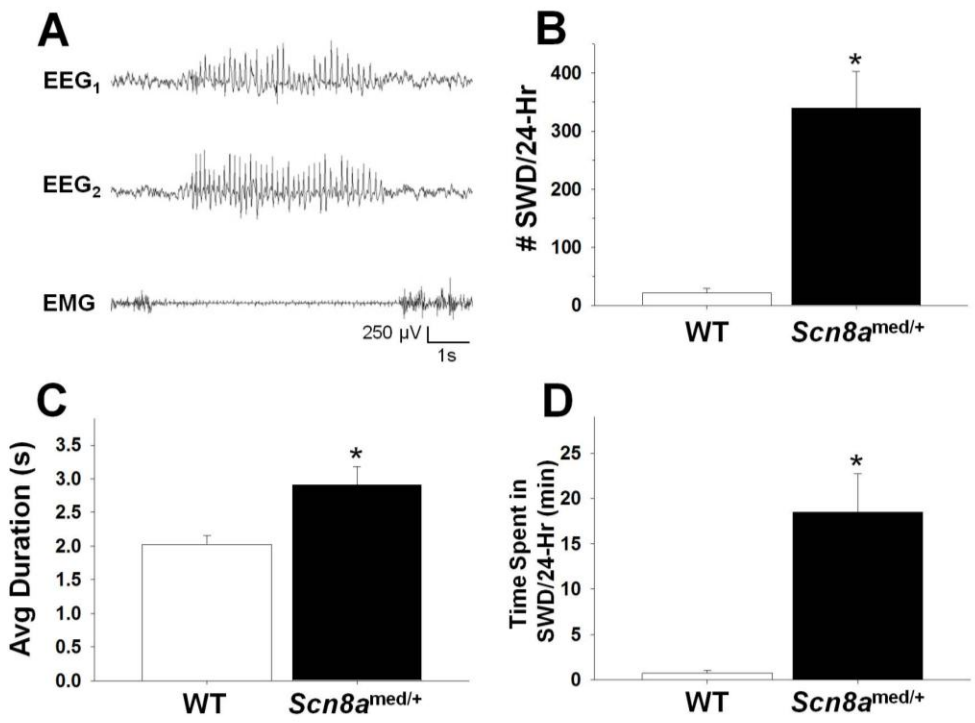

Figure 2.1 SWD activity over a 24-hour baseline period. (A) Representative SWD in *Scn8a*$^{med/+}$ mutant. EEG1 and EEG2 represent cortical activity from the right and left hemispheres, respectively. SWD are present in both hemispheres and are associated with lack of muscle movement (EMG). **(B)** *Scn8a*$^{med/+}$ mice have more SWDs in a 24-hour period than WT mice: $t_{(16)}$=-4.457, *p<0.001. **(C)** The SWDs exhibited by *Scn8a*$^{med/+}$ mice last longer than SWDs in WT mice: $t_{(14)}$=-2.402, *p<0.05. **(D)** *Scn8a*$^{med/+}$ mice spend significantly more time experiencing SWDs in a 24-hour period than WT mice:

initial suppression of SWD activity while the animals were in the restraint tubes (0-20 min), there was a significant increase in SWD activity between 40 and 80 min after the onset of the stressor (**Fig. 2.2**C). Given the appearance of an initial suppression of SWD activity, we determined the average latency to the first absence seizure following stress exposure and found *Scn8a*$^{med/+}$ mice have a significantly shorter latency, 24 min, to the first SWD event compared to WT littermates (**Fig. 2.2**D).

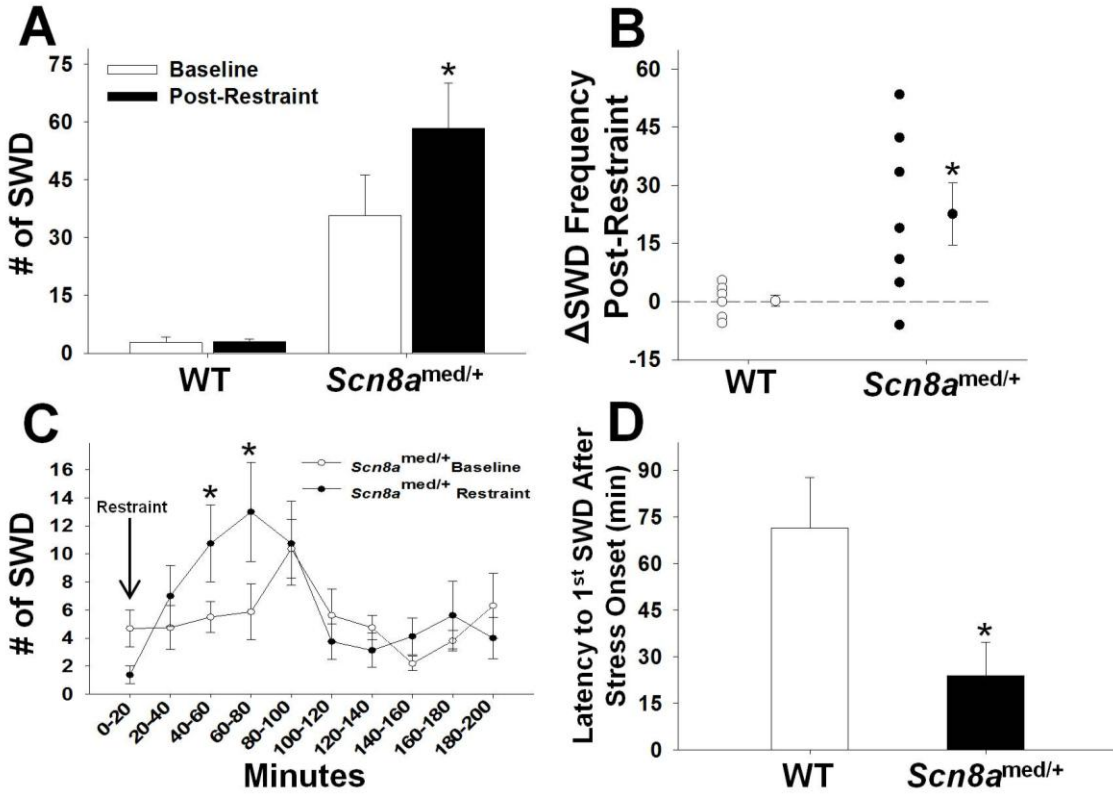

Figure 2.2 SWD activity in response to a 20-minute acute restraint stress. (A) *Scn8a*$^{med/+}$ mice have significantly more SWDs in the 2-hour period following restraint stress compared to the same 2-hour period at baseline, while WT mice show no increase in number of SWDs following restraint stress (genotype main effect: $F_{(1,12)}$=17.969, p<0.01; stress main effect: $F_{(1,12)}$=7.7.36, p<0.05; genotype x stress interaction effect: $F_{(1,12)}$=7.448, p<0.05). *p<0.05 vs. Baseline within genotype, Tukey post hoc test. **(B)** *Scn8a*$^{med/+}$ mice have a significant change in SWD frequency after restraint stress compared to WT mice: $t_{(12)}$=-2.729, *p<0.05. **(C)** SWD activity in *Scn8a*$^{med/+}$ mice in 20-minute intervals beginning with the restraint stress (min 0-20) and ending 3 hours after the end of the stressor. Following an initial suppression of SWD activity while in the restraint tube, *Scn8a*$^{med/+}$ mice show a significant increase in SWDs 40 to 80 minutes post-restraint (time main effect: $F_{(9,63)}$=3.818, p<0.001; time x stress interaction effect: $F_{(9,63)}$=2.619, p<0.05). *p<0.05 vs. Baseline, Tukey post hoc test. **(D)** After the onset of a stressor, *Scn8a*$^{med/+}$ mice show a shorter latency to the first post-stress SWD than WT mice: $t_{(11)}$=6.321, *p<0.001. Error bars represent SEM. n=6-8 per group.

2.4.3 Acute stress affects SWD rhythm in *Scn8a*$^{med/+}$ mice for up to 60 hours after the stress exposure

Upon analyzing SWD frequency during 24 h of continuous baseline recordings, we observed that the *Scn8a*$^{med/+}$ mice, but not WT littermates, have a 24-h diurnal rhythm of absence seizures, with a significant peak of SWD activity between 5-7 pm, coinciding with the end of the light period (**Fig. 2.3**A). As illustrated in a dot plot showing individual SWDs experienced by each of the 7 mice from 5-9 pm, this peak in SWD activity was followed by a sharp decrease in baseline SWD activity at 7 pm (**Fig. 2.3**B). Interestingly, the decrease in SWD activity at 7 pm was not seen the evening of the same day that a 20-min acute restraint stress was administered during the morning (8:00-10:00 am) (**Fig. 2.3**C). To examine this finding in more detail, post-restraint SWD frequency in *Scn8a*$^{med/+}$ mice was analyzed in 2-h intervals from 1 pm to 7 am. Analysis showed the mice that experienced restraint stress displayed an altered SWD pattern, with the peak of seizure activity extending beyond 5-7 pm, resulting in increased SWD activity from 7-11 pm (**Fig. 2.3**D). As the morning restraint was performed between 8:00-10:00 am, these changes in SWD activity occurred almost 12 h following stress exposure. We examined a separate group of animals to determine how long these changes persisted. We found that the evening SWD rhythm was still disrupted 36 h post-restraint, and did not return to the normal baseline pattern until the third evening, 60 h post-restraint (data not shown).

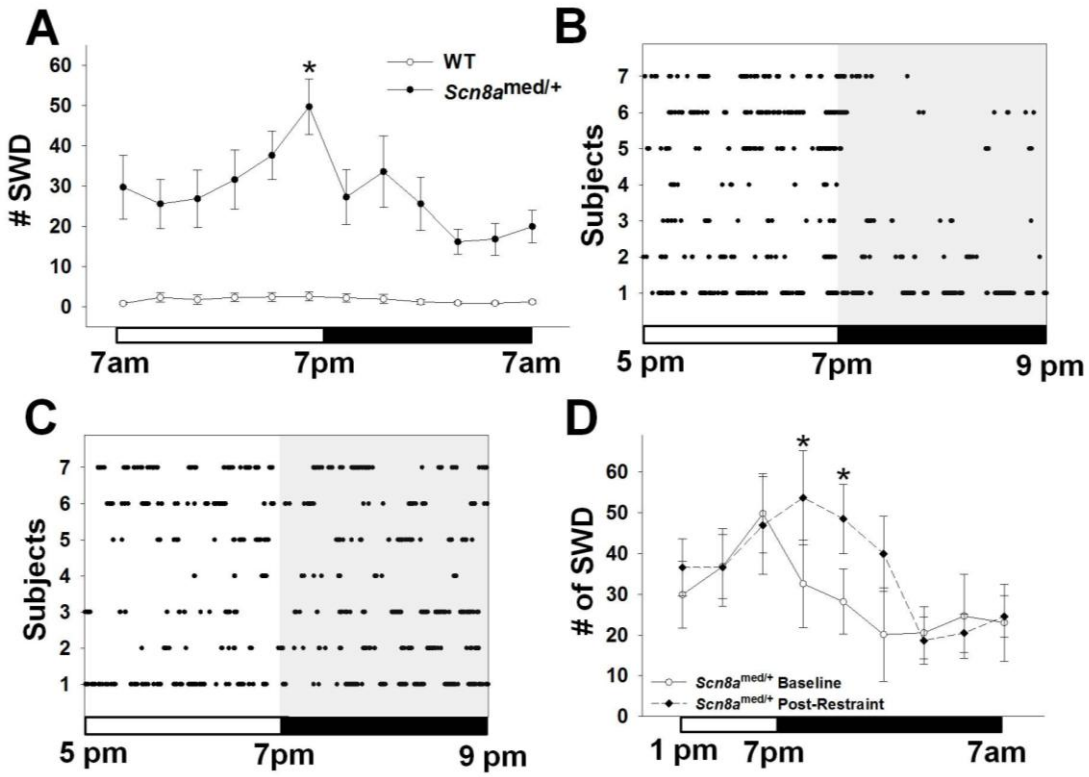

Figure 2.3 Long-lasting SWD response to acute stress. (A) *Scn8a*$^{med/+}$ mice show a 24-hour rhythm in SWD frequency when analyzed in 2-hour intervals, with the peak in activity occurring between 5-7 pm (genotype main effect: $F_{(1,16)}$=19.866, p<0.001; time main effect: $F_{(11,176)}$=4.990, p<0.001; genotype x time interaction effect: $F_{(11,176)}$=4.054, p<0.001). *p<0.05 vs. other time points except 3-5 pm within genotype, Tukey post hoc test. **(B,C)** Dots represent individual SWDs by *Scn8a*$^{med/+}$ mice from 5-9 pm during both a baseline period **(B)** and the evening following a morning exposure to acute restraint stress **(C)**. The sharp drop-off of SWD activity at 7 pm disappears following the morning restraint stress. **(D)** The morning restraint stress alters the evening rhythm of SWD activity in *Scn8a*$^{med/+}$ mice by shifting and broadening the peak from 5-7 pm to 7-11 pm (time main effect: $F_{(8,48)}$=4.241, p<0.001: time x stress interaction effect: $F_{(8,48)}$=2.524, p<0.05). *p<0.05 vs. Baseline, Tukey post hoc test. Error bars represent SEM. n=7-8 per group.

2.4.4 Acute stress reduces latencies to and increases severity of picrotoxin-induced and kainic acid-induced seizures in *Scn8a*$^{med/+}$ mice

To determine the effect of acute stress on seizure thresholds, we first subjected *Scn8a*$^{med/+}$ mice and WT littermates to a 20-min acute restraint stress immediately before injection with picrotoxin (5 mg/kg, i.p.). We had previously established that, under normal conditions, *Scn8a*$^{med/+}$ mice have elevated thresholds to generalized tonic-clonic seizures (GTCSs) induced by flurothyl and reduced seizure severity in response to kainic acid (Martin *et al.* 2007). Consistent with this finding, under unstressed conditions the mutants exhibited increased latency to picrotoxin-induced GTCS (**Fig. 2.4**A, white bars) and reduced seizure severity compared to WT littermates (**Fig. 2.4**B, white bars). Furthermore, consistent with our previous findings, significantly fewer mutants in the unstressed group progressed to a GTCS when compared to WT littermates (**Fig. 2.4**C, white bars).

However, acute restraint stress affected GTCS latencies differently in *Scn8a*$^{med/+}$ mice than in WT littermates (**Fig. 2.4**A). While the latency in WT animals increased, the latency in *Scn8a*$^{med/+}$ mice decreased, resulting in a significant stress-genotype interaction ($F_{(1,36)}$=4.594, $p<0.05$). Although the magnitude of the change was not statistically significant in either genotype, there was a trend towards decreased latency in the *Scn8a*$^{med/+}$ mice (Tukey, p=0.076). Furthermore, acute stress significantly increased the severity of the seizures in the *Scn8a*$^{med/+}$ mice, while the increase in the WT animals was not significant (**Fig. 2.4**B), and stress increased the number of *Scn8a*$^{med/+}$ mice that progressed to the GTCS stage (**Fig. 2.4**C). Stress also increased the percentage of

Scn8a$^{med/+}$ mice having seizures lasting longer than 15 min, whereas acute stress did not affect seizure duration in WT mice (**Fig. 2.4**D).

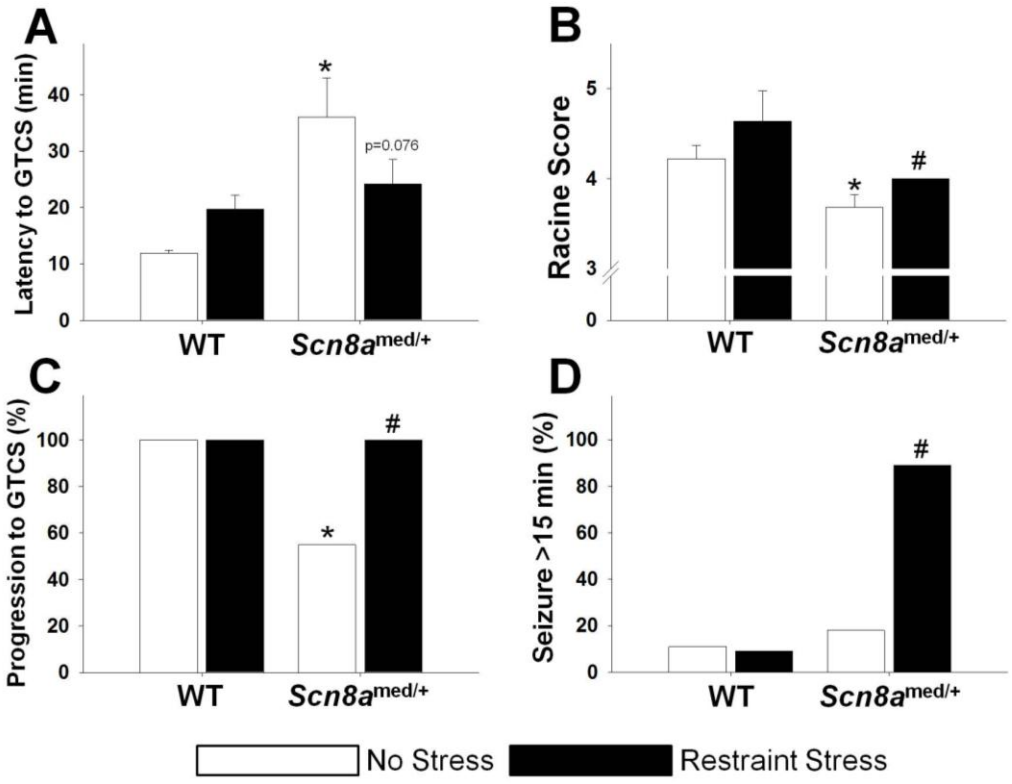

Figure 2.4 Effect of stress on picrotoxin-induced seizures. (A) Under normal conditions, *Scn8a*$^{med/+}$ mice have increased picrotoxin-induced seizure thresholds compared to WT littermates (genotype main effect: $F_{(1,36)}$=9.761, p<0.01). However, while restraint stress increases thresholds in WT mice, it decreases thresholds in *Scn8a*$^{med/+}$ mice (genotype x stress interaction effect: $F_{(1,36)}$=4.594, p<0.05). *p<0.05 vs. WT littermates within condition, Tukey post hoc test. **(B)** Exposure to restraint stress increases seizure severity as measured by the Racine score, but the increase is only significant in *Scn8a*$^{med/+}$ mice: Mann-Whitney, U=21, *p<0.05 vs. WT within condition; Mann-Whitney, U=27, #p<0.05 vs. No Stress within genotype. **(C)** Under normal conditions, fewer *Scn8a*$^{med/+}$ mice progress to a GTCS than WT mice, but restraint stress significantly increases the percentage of *Scn8a*$^{med/+}$ mice progressing to a GTCS: Fisher Exact, *p<0.05 vs. WT within condition; Fisher Exact, #p<0.05 vs. No Stress within genotype. **(D)** Acute restraint stress results in an increase in the percent of *Scn8a*$^{med/+}$ mice experiencing seizures lasting longer than 15 minutes: Fisher's Exact, #p<0.01 vs. No Stress within genotype. Error bars represent SEM. n=9-11 per group.

To measure the effect of stress on thresholds to seizures induced by a different mechanism, mice were subjected to 20-min acute restraint stress immediately prior to injection with kainic acid (30 mg/kg, i.p.). We were able to replicate our previous findings of increased latency to a GTCS in unstressed *Scn8a*$^{med/+}$ mice (**Fig. 2.5**C, white bars) and decreased seizure severity in unstressed mutant mice (**Fig. 2.5**D, white bars) compared to WT littermates following kainic acid administration (Martin *et al.* 2007). We were also able to show that the unstressed mutants have an increased latency to Stage 3 seizure activity (unilateral forelimb clonus) compared to unstressed WT littermates (**Fig. 2.5**B, white bars).

Following acute stress, there was a significant effect of stress on both Stage 2 (automatisms) and Stage 3 (unilateral forelimb clonus) seizure latencies. Stress significantly reduced the latency to Stage 2 in *Scn8a*$^{med/+}$ mice (**Fig. 2.5**A) and showed a trend for reducing the latency to Stage 3 in the mutants (**Fig. 2.5**B). However, acute stress affected neither the latency to Stage 6 (GTCS) nor the Racine score for seizure severity (**Figs. 2.5**C,D). Overall, these data show that acute restraint stress worsens seizure outcome by decreasing seizure latency and increasing seizure severity in *Scn8a*$^{med/+}$ mice following administration of a chemiconvulsant, although stress had more of an effect on picrotoxin-induced seizures than on kainic acid-induced seizures. In both cases, stress affected mutants differently than WT littermates.

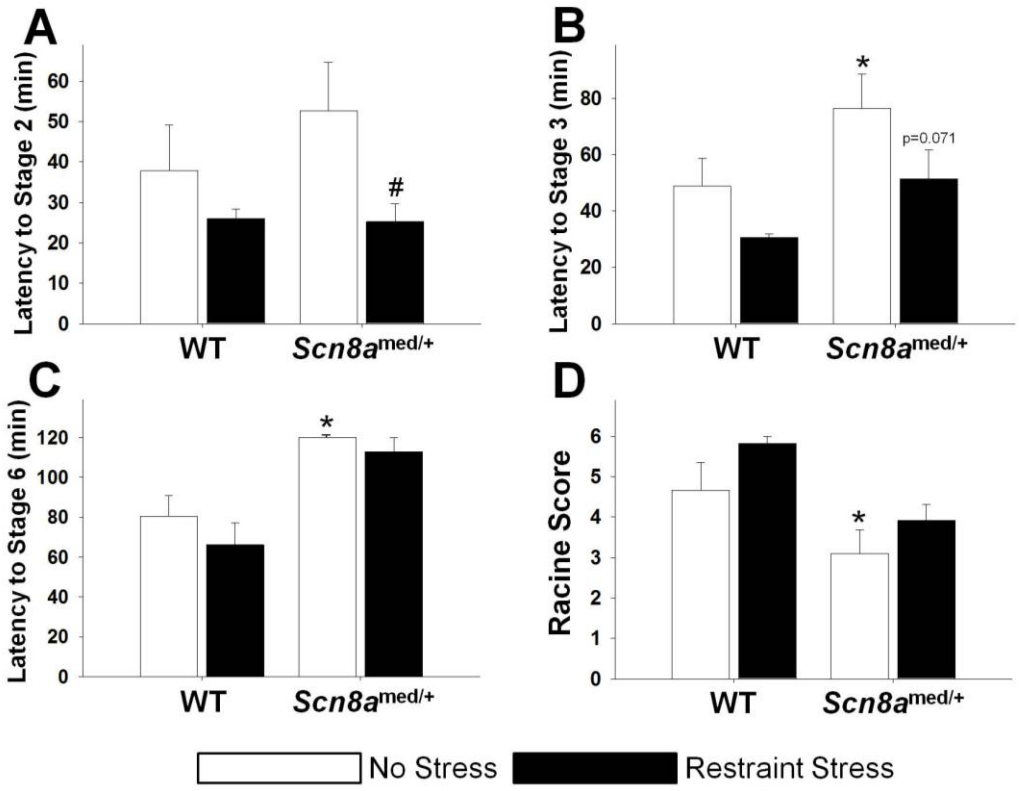

Figure 2.5 Effect of stress on kainic acid-induced seizures. (A) Stress significantly reduced the latency to Racine Stage 2 (automatisms) in the *Scn8a*$^{med/+}$ mice, but not in their WT littermates, following injection with kainic acid (stress main effect: $F_{(1,41)}$=5.343, p<0.05). #p<0.05 vs. No Stress within genotype, Tukey post hoc test. **(B)** Under unstressed conditions, *Scn8a*$^{med/+}$ mice have a higher threshold to a kainic acid-induced Stage 3 (unilateral forelimb clonus) seizure than WT mice (genotype main effect: $F_{(1,40)}$=6.399, p<0.05). *p<0.05 vs. WT within condition, Tukey post hoc test. Following exposure to stress, there is a trend for a reduced latency to Stage 3 in the *Scn8a*$^{med/+}$ mice, but not in WT mice (stress main effect: $F_{(1,40)}$=5.106, p<0.05). **(C)** *Scn8a*$^{med/+}$ mice have an increased latency to Stage 6 (GTCS) compared to WT mice under unstressed conditions (genotype main effect: $F_{(1,40)}$=24.927, p<0.001). *p<0.05 vs. WT within condition, Tukey post hoc test. Restraint stress does not affect the latency to GTCS in either genotype. **(D)** *Scn8a*$^{med/+}$ mice have reduced seizure severity compared to WT mice under unstressed conditions (Mann-Whitney, U=26, *p<0.05 vs. WT within condition), but stress does not significantly affect seizure severity following kainic acid administration. Error bars represent SEM. n=10-12 per group.

2.4.5 *Scn8a*$^{med/+}$ Mice have normal HPA axis activity

We examined the diurnal HPA axis activity by measuring corticosterone (CORT) levels at the beginning of the light (7 am) and dark (7 pm) phases. Both mutants and WT littermates have an intact diurnal variation in CORT (**Fig. 2.6**A). We tested the HPA axis response to an acute stressor by measuring CORT response at baseline and at 5 different time points following an acute 20-min restraint stress. Stress exposure in both genotypes resulted in an immediate surge in CORT levels, followed by a gradual decline over the next 2 h (**Fig. 2.6**B). Thus, *Scn8a*$^{med/+}$ mice display a normal HPA axis response to an acute stressor, as well as normal negative feedback regulation to terminate the stress response.

2.4.6 Acute stress affects HPA axis circadian activity in *Scn8a*$^{med/+}$ mice for up to 12 hours after the stress exposure

To determine whether acute restraint stress could have a long-term effect on HPA axis circadian activity, we first sampled blood from a group of animals at 7 pm to establish the normal CORT baseline levels. A week later, we took a basal blood draw immediately before subjecting each mouse to a 20-min acute restraint stress that occurred between 8-9 am, followed by a final blood draw at 7 pm of the same day. Consistent with the data presented in **Fig. 2.6**B, we found no differences in basal CORT levels between *Scn8a*$^{med/+}$ mice and their WT littermates (**Fig. 2.6**C). However, following the restraint stress, the 7 pm CORT levels in the *Scn8a*$^{med/+}$ mice were significantly higher than both

the baseline 7 pm levels in *Scn8a*$^{med/+}$ mice and the post-stress 7 pm levels in the WT littermates (**Fig. 2.6**C).

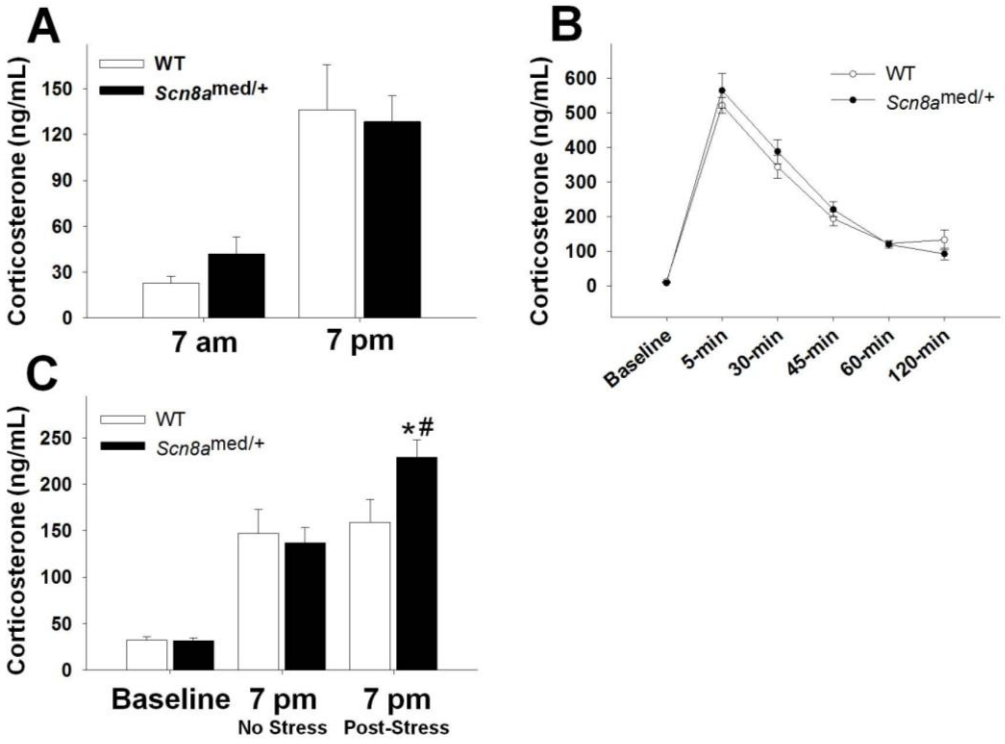

Figure 2.6 HPA axis function. (A) *Snc8a*$^{med/+}$ mice have normal diurnal fluctuations in plasma corticosterone levels, with similar corticosterone levels as WT mice during both the nadir (7 am) and peak (7 pm) of the circadian HPA axis rhythm (time main effect: $F_{(1,16)}$=34.011, p<0.001). **(B)** Plasma corticosterone levels before (baseline) and at 5, 30, 45, 60, and 120 min following a 20-minute restraint stress. There were no differences in the HPA axis response between *Scn8a*$^{med/+}$ mice and WT mice (time main effect: $F_{(5,130)}$=100.337, p<0.001). **(C)** Although there are no basal differences in plasma corticosterone levels prior to the stressor (baseline) between genotypes ($t_{(20)}$=0.216, p=0.831), morning exposure to acute restraint stress increases corticosterone levels at 7 pm in *Scn8a*med/+ mice, but not in WT littermates (stress main effect: $F_{(1,20)}$=14.505, p<0.01; stress x genotype interaction effect: $F_{(1,20)}$=8.605, p<0.01). *p<0.05 vs. WT within Post-Stress condition and #p<0.05 vs. No Stress condition within genotype, Tukey post hoc test. Error bars represent SEM. n=9-12 per group.

2.4.7 *Scn8a*$^{med/+}$ mice show increased anxiety-like behavior

The first behavioral difference we observed was that *Scn8a*$^{med/+}$ mice struggled significantly less while in the restraint tubes than WT littermates (**Fig. 2.7**A). This result prompted us to further examine the behavior of *Scn8a*$^{med/+}$ mice in other behavioral tasks. We exposed mice to predator odor using the synthetic fox feces compound TMT dissolved in ethanol. Analysis of freezing showed that, while both genotypes exhibited increased freezing in the presence of the predator odor, the *Scn8a*$^{med/+}$ mice froze significantly more than the WT littermates (**Fig. 2.7**B). There were no differences in freezing behavior between genotypes in the absence of the predator odor (**Fig. 2.7**B). Examination of rearing, a characteristic exploratory behavior in rodents that should decrease in situations of perceived danger, revealed that both mutants and WT mice reared significantly less in the presence of the odor as expected (**Fig. 2.7**C). Interestingly, in the no-odor (vehicle) condition, the *Scn8a*$^{med/+}$ mice reared significantly less than the WT littermates, suggesting reduced exploratory behavior in the mutants (**Fig. 2.7**C).

To further characterize exploratory behavior, animals were next placed into a novel environment and observed for grooming and exploratory behaviors. Increased grooming has been linked to increased levels of anxiety, as grooming is a displacement response in stress-inducing situations (Espejo 1997). *Scn8a*$^{med/+}$ mice were observed to engage in significantly more grooming episodes than the WT littermates (**Fig. 2.7**D). *Scn8a*$^{med/+}$ mice also exhibit fewer stretch-attends, a risk-assessment behavior used by a rodent in exploration of a novel environment (Choleris *et al.* 2001), than WT littermates (**Fig. 2.7**D). *Scn8a*$^{med/+}$ mice also reared significantly less than WT littermates, in

agreement with observations during the vehicle condition of the predator odor experiment (**Fig. 2.7**D). Taken together, the reduced struggling, increased freezing, increased grooming, and decreased exploratory activities indicate a higher level of anxiety in the *Scn8a*$^{med/+}$ mice.

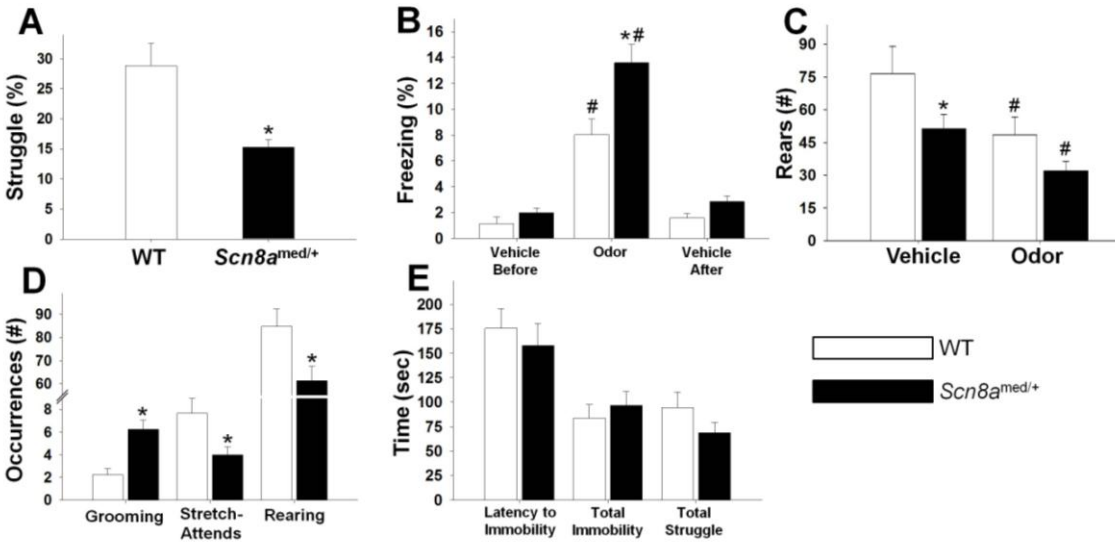

Figure 2.7 Behavior in stressful situations. (A) *Scn8a*$^{med/+}$ mice struggled less in the restraint tubes during the 20-minute period of restraint stress compared to WT littermates: $t_{(12)}$=3.504, *p<0.01. **(B)** In response to predator odor exposure, both *Scn8a*$^{med/+}$ mice and WT mice showed increased freezing, but the *Scn8a*$^{med/+}$ mice froze significantly more than their WT littermates (genotype main effect: $F_{(1,24)}$=10.447, p<0.01; odor exposure main effect: $F_{(2,48)}$=88.992, p<0.001; genotype x odor exposure interaction effect: $F_{(2,48)}$=5.729, p<0.01). *p<0.05 vs. WT within condition and #p<0.05 vs. Vehicle (before and after) within each genotype, Tukey post hoc test. **(C)** Under control conditions, *Scn8a*$^{med/+}$ mice rear less than WT mice, and exposure to predator odor significantly reduces rearing in both genotypes (odor exposure main effect: $F_{(1,24)}$=21.454, p<0.001); trend for genotype main effect: $F_{(1,24)}$=3.803, p=0.063). *p<0.05 vs. WT within condition and #p<0.05 vs. Vehicle within genotype, Tukey post hoc test. **(D)** In response to novelty exposure, *Scn8a*$^{med/+}$ mice show increased grooming and reduced exploratory behaviors (stretch-attends and rearing) compared to their WT littermates (grooming: $t_{(22)}$=-4.119, *p<0.001; stretch-attends: $t_{(22)}$=2.454, *p<0.05; rearing: $t_{(22)}$=2.432, *p<0.05). **(E)** There were no differences between *Scn8a*$^{med/+}$ mice and WT mice in forced swim test measures. Error bars represent SEM. n=7-13 per group.

We also measured depressive-like behavior using the Porsolt forced swim test and found no significant differences between *Scn8a*$^{med/+}$ mice and WT littermates in any of the measures tested (**Fig. 2.7**E), indicating that *Scn8a*$^{med/+}$ mice do not exhibit depressive-like behaviors as measured by this test.

2.5 Discussion

The present study provides important new information linking a specific monogenic mutation to diurnal seizure activity and demonstrating the effects of an acute stressor on the diurnal rhythm of seizures. Specifically, the principal findings of the present study are: (1) Acute stress can have both short- and long-term effects on spontaneous absence seizure frequency, (2) Acute stress reduces seizure thresholds and increases the severity of chemically induced seizures in *Scn8a*$^{med/+}$ mice, but not in WT littermates, (3) Acute stress affects HPA axis diurnal rhythm in *Scn8a*$^{med/+}$ mice, but not in WT littermates, and (4) The *Scn8a*$^{med/+}$ mutation is sufficient to increase anxiety-like behaviors in response to mildly stressful situations. In contrast to much of the previous research on the relationship between stress and epilepsy that mostly used wild-type animal models with normal brain and stress system development (Sawyer and Escayg 2010), the current study demonstrates that an inherited sodium channel mutation can alter the seizure response to stress. Furthermore, because sodium channels play a major role in the regulation of neuronal excitability, and altered neuronal excitability is believed to underlie a number of neurological and neuropsychiatric disorders, findings from this study may have broader implications for understanding the impact of stress on disease processes.

Interestingly, a brief, 20-minute restraint stress in the morning resulted in a transient increase in SWD activity, yet also had long-lasting effects on the circadian rhythm of SWD activity. At the transition from the light to the dark period on the same day as the stressor, we found that *Scn8a*$^{med/+}$ mice had an extended period of elevated SWD activity lasting well into the evening, an altered rhythm that did not resume its normal pattern until 60 hours following the stress exposure. In humans, daily stressful events and perceived stress levels are reported to be strongly associated with seizures over both the short-term and up to 24 hours later (Temkin and Davis 1984), consistent with our findings. Wistar Albino Glaxo rats from Rijswijk (WAG/Rij), another animal model of absence epilepsy, also demonstrate a baseline 24-hour rhythm in SWD occurrence, with a maximal peak of seizure activity at the end of the light phase (Smyk *et al.* 2011), consistent with our results. However, the current study is the first to describe the long-term effects of acute stress on SWD activity in an animal model of absence epilepsy. Discovery of this long-term effect now gives us the opportunity to investigate how stress works to prime the epileptic brain for seizure activity long after the stressor has passed.

Interestingly, the diurnal SWD activity in our mice matches the diurnal HPA axis activity: we saw a peak in SWD activity from 5-7 pm corresponding with increased CORT levels at 7 pm. Furthermore, the time course of HPA axis activity after an acute stressor follows a similar pattern as the SWD activity, with both responses increasing soon after stress exposure and returning to baseline about two hours later. While an argument could be made that the increased seizure activity is contributing to an increase in HPA axis activity, our results are more consistent with the converse relationship of

increased HPA axis activity leading to increased SWD activity. The elevation in CORT levels following the acute stress precedes the increase in seizure activity by 20 minutes or more, suggesting that the increase in HPA axis activity precedes the short-term increase in SWD activity. In a study with WAG/Rij rats, direct injections of CORT increased SWD activity 15-30 minutes after injection (Schridde and van Luijtelaar 2004), further evidence that it may be the increase in CORT that is driving the increase in SWD activity. The immediate anti-convulsant response to stress is likely meditated by the rapid noradrenergic stress response which precedes the longer-lasting genomic actions of the HPA axis response (Giorgi *et al.* 2004). CORT is also known to alter sodium currents by changing voltage-dependent activation and inactivation and recovery from inactivation in hippocampal neurons (Werkman *et al.* 1997), as well as voltage-dependent sodium conductances (Joels 1997), providing a possible mechanistic explanation for how HPA axis activity may induce seizure activity. However, further investigation into the relationship between seizure activity and HPA axis activity in the *Scn8a*$^{med/+}$ mutants is needed to establish whether the link between the immediate increase in HPA axis activity and the short-term increase in seizure activity is directly causative, or whether these changes may result from a common upstream mediator.

Our study also provides evidence of a link between increased HPA axis activity and long-lasting alterations in SWD activity. Following a morning exposure to an acute stressor, we found that 7 pm CORT levels in the mutants were elevated above baseline, and the normal evening rhythm of SWD activity was altered. There is evidence that seizure activity can have long-term effects on HPA axis activity, possibly resulting in a positive feedback loop that could raise the chances of spontaneous seizures in the future.

In seizure-sensitive gerbils, corticotropin-releasing hormone (CRH) immunoreactivity following a seizure does not return to pre-seizure levels until 24 hours later (An *et al.* 2003), and pilocarpine-induced status epilepticus (SE) can increase CRH gene expression significantly for up to a day after the SE (Wu *et al.* 2012). In both cases, CRH activity is altered, and CRH is consistently found to be pro-convulsant (Sawyer and Escayg 2010). Considering that only the *Scn8a*$^{med/+}$ mutants, and not the wild-type littermates, show a long-lasting change in HPA axis activity after an acute stressor, altered sodium channel function may well mediate such long-lasting HPA axis-seizure feedback loops.

Given the proposed link between hyperemotionality and epilepsy and the possible involvement of the HPA axis in both phenomena, we also assessed the behavioral response of *Scn8a*$^{med/+}$ mice to mildly stressful situations. We found that *Scn8a*$^{med/+}$ mice show higher levels of anxiety-like behavior, characterized by decreased struggling during restraint stress, increasing freezing, increased grooming, and decreased exploratory behavior, but they did not show any measure of depressive-like behavior in the forced swim test. These behavioral changes are unlikely to be due to elevated SWD activity in response to the stressful nature of the task, as we have also shown that SWD activity is suppressed for an average of 24 minutes following the onset of a stressor, and all of the behavioral tasks were completed in 15 minutes or less. Furthermore, the mutants exhibited an increase in grooming in response to novelty and no difference in struggling during the forced swim test, behaviors that would be inconsistent with SWD-induced behavioral arrest. Our behavioral findings are consistent with other reported findings, and increased anxiety-like behavior in animal models of epilepsy appears to be highly reproducible (Heinrichs 2010). For example, the WAG/Rij rats show increased agitation,

decreased exploration, increased grooming, and hyperlocomotion in response to novelty stress (Midzyanovskaya *et al.* 2005). Intriguingly, anxiety levels in WAG/Rij rats are positively correlated with the propensity for SWD activity, with rats having the greatest number of SWDs showing the highest anxiety levels (Midzyanovskaya *et al.* 2005). Both WAG/Rij rats and the Genetic Absence Epilepsy Rats from Strasbourg (GAERS) also show depressive-like behaviors (Jones *et al.* 2008; Sarkisova and van Luijtelaar 2011), whereas the *Scn8a*$^{med/+}$ mutants did not differ from wild-type littermates in depressive measures tested. Linkage studies in the GAERS and WAG/Rij rats show that the genes linked with SWD activity in these two models are on different chromosomes, suggesting different genetic causes for SWD activity and perhaps even the behavioral abnormalities seen in these models (Gauguier *et al.* 2004; Rudolf *et al.* 2004). The *Scn8a*$^{med/+}$ model, on the other hand, allows us to speculate that a VGSC mutation might preferentially affect systems involved in the anxiety response. Evidence provided by Mirza et al. (2005) shows that VGSCs can indeed mediate the anxiety response, while McKinney et al. (2008) saw increases in anxiety-like behaviors in the *Scn8a*^{tg} mutant, and we previously found minor changes in anxiety-like behavior in the *Scn8a*$^{med\text{-}jo}$ mutant (Papale *et al.* 2010). Increased levels of anxiety are also seen in mutants with deficits in another VGSC gene, *Scn1a* (Han *et al.* 2012; Ito *et al.* 2012). The evidence from these experiments and the results from the current study suggest that *Scn8a* and VGSCs in general do play a role in anxiety-like behaviors.

In contrast to the stress-induced changes discussed above, baseline HPA axis activity in the mutants is not altered; however, this finding is consistent with literature showing that humans with generalized anxiety disorders, when analyzed separately from

those with comorbid depression or post-traumatic stress disorder, often show no changes in baseline HPA axis activity (Vreeburg *et al.* 2010; Staufenbiel *et al.* 2012). Considering that an anxiety disorder is characterized by a mental and physiological overreaction to a stressful situation (Hoehn-Saric and McLeod 2000), it is likely that at baseline in a non-stressed state, individuals appear physiologically normal. We found the CORT response to an acute stressor was the same in the *Scn8a*$^{med/+}$ mutants as in WT littermates, suggesting a normal initial response to a stressor, but the mutants showed altered CORT levels up to 12 hours after the stressor. Thus, abnormalities in the HPA axis function of *Scn8a*$^{med/+}$ mice may not become apparent until after the system is challenged by a stressful situation.

In contrast to the spontaneous SWD activity, *Scn8a*-deficient mice show elevated thresholds to induced seizures (Martin *et al.* 2007; Blumenfeld *et al.* 2009), a finding also confirmed by the present study. Interestingly, we found that acute stress affects chemically induced seizures in *Scn8a*$^{med/+}$ mice differently than in WT littermates. Specifically, acute stress increased thresholds to picrotoxin-induced seizures in WT littermates, but decreased thresholds in *Scn8a*$^{med/+}$ mice. Acute stress also increased seizure severity, the number of animals progressing to a GTCS, and the number of animals experiencing seizures lasting longer than 15 minutes in the *Scn8a*$^{med/+}$ mutants, but not the WT littermates. Given that seizure activity, anxiety, and the stress response all involve limbic structures, we propose that the hyperemotionality seen in the *Scn8a*$^{med/+}$ mice could be working in a feed-forward manner to influence the seizure response to a stressor. During the 20 minutes that the mice are in the restraint tubes prior to seizure induction, they are not only experiencing a stress response in terms of increased

corticosterone levels, but they are also experiencing an anxiety response. The behavioral response to the stressor precedes the altered seizure response, suggesting that the alterations in anxiety-like behaviors may be driving the alterations in the seizure response to stress and elevated CORT, which then lead to long-lasting changes in HPA axis activity that in turn influence future seizure activity.

The results of this study support a role for *Scn8a* in anxiety-like behaviors. In addition, this study suggests a connection between altered anxiety-like behaviors, prolonged changes in HPA axis circadian activity, and spontaneous seizure activity in response to a stressor. Furthermore, previous work examining the role of stress in multifactorial models of epilepsy, such as the epilepsy (EL) mouse and the WAG/Rij rat, have shown that stress or fear can trigger spontaneous seizure activity (Seyfried *et al.* 1999; Tolmacheva *et al.* 2012). While these are validated models of genetic epilepsy, it is difficult to ascribe the results of those experiments to a single mechanism or gene. In contrast, we have shown that acute stress worsens seizure outcome and alters behavior in *Scn8a*$^{med/+}$ mice. The differences between mutants and WT animals suggest that *Scn8a*, by altering neuronal excitability, plays a role in the stress response, highlighting the need for further research into the interplay between stress, anxiety, and seizures in genetically predisposed animal models, as well as future research into the role of VGSCs in the stress response. A better understanding of the impact of *Scn8a* on the stress response may give us insight into the mechanisms of stress-evoked disorders and point the way to new therapeutic targets for the treatment of epilepsy.

2.6 Acknowledgements

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CHAPTER 3: EARLY LIFE EXPERIENCE ALTERS ADULT SEIZURE OUTCOME AND BEHAVIOR IN A VOLTAGE-GATED SODIUM CHANNEL (*SCN8A*) MUTANT

3.1 Abstract

Early life experience is known to permanently modify the activity of the hypothalamic-pituitary-adrenal (HPA) axis and to result in enduring behavioral effects. The purpose of this study was to examine the effect of early life experience on seizure outcome and behavior in adult mice with mutations in the voltage-gated sodium channel (VGSC) gene *Scn8a*. The model of early life experience chosen was the maternal separation (MS) paradigm in which pups were separated from their dam for 3 hours per day from P2 until P14. Intriguingly, the pups in the MS groups received increased levels of maternal care and demonstrated improved seizure outcome in adulthood in response to chemically induced seizures. In addition to raising seizure thresholds, MS also decreased corticosterone (CORT) response levels in females and reduced sex differences in open field behaviors. MS did not seem to affect spontaneous SWD activity in the *Scn8a* mutants. Our results also indicate that some phenotypic characteristics of an *Scn8a* mutant are dependent on early life experience. Under the conditions of this study, male *Scn8a* mutants exhibited a higher CORT response to an acute stressor and an increase in depressive-like behavior, phenotypes not previously seen in *Scn8a* mutants. We also describe multiple sex effects within the control group, such as the fact that females have higher thresholds to induced seizures, higher CORT levels, and are less active in the open field. This study reveals a complex interplay of factors with interactions between MS and genotype, MS and sex, and genotype and sex. Overall, these findings suggest that early life experience can have different effects dependent on both genetics and sex. Ultimately, our data show that a VGSC mutation in conjunction with early life experience can alter

adult seizure outcome and behavior and could, in part, provide an explanation for the diverse array of phenotypes seen in epilepsy patients.

3.2 Introduction

As described previously, in the central nervous system, $Na_v1.6$ (encoded by the gene *SCN8A*) is the most widely expressed VGSC, clustered in high density at axonal initial segments and nodes of Ranvier, and found in both presynaptic and postsynaptic membranes (Caldwell et al. 2000; Boiko et al. 2003). $Na_v1.6$ plays a major role in carrying subthreshold currents, namely persistent current and resurgent current (Raman et al. 1997), and the electrophysiological properties of $Na_v1.6$ make it especially suited for the sustained repetitive firing of neurons (Van Wart and Matthews 2006; Chen et al. 2008). Our lab has demonstrated that while *Scn8a*$^{med/+}$ mice are resistant to chemically induced seizures (Martin *et al.* 2007), they exhibit frequent spontaneous spike wave discharges (SWDs) characteristic of absence epilepsy (Papale *et al.* 2009).

Given the paroxysmal nature of seizures, it is hypothesized that endogenous or environmental triggering factors are responsible for seizure initiation. Epilepsy patients consistently self-report stress as being the most common trigger for seizures (Spatt et al. 1998; Haut et al. 2003; Nakken et al. 2005; Haut et al. 2007; Sperling et al. 2008). We recently investigated the role of acute stress as a trigger for seizures in *Scn8a*$^{med/+}$ mutants (see Chapter 2). We found that a VGSC mutation can alter the behavioral response to stress and interact with the stress response to influence seizure outcome (Sawyer et al. 2014). Specifically, we found that an acute stressor was able to increase spontaneous

SWD activity over both the short-term and up to 60 hours following the stress exposure (Sawyer et al. 2014). Acute stress also resulted in an increase in the severity and duration of chemically-induced seizures, and we found that *Scn8a*$^{med/+}$ mutants exhibited anxiety-like behaviors (Sawyer et al. 2014). While the finding that an acute stressor could affect spontaneous SWD activity for several days was surprising, exposure to an acute stress does not permanently change the functioning of the hypothalamic-pituitary-adrenal (HPA) axis, a major physiological stress response system. Therefore, we wondered how long-term alterations to stress reactivity would affect the seizure phenotype and behavior in the *Scn8a*$^{med/+}$ mutants, which led to the design of the current study.

Long-lasting changes in stress reactivity can be achieved by manipulating the early life experience or environment. A large amount of literature exists showing that early life experiences can have a profound influence on adult behavior and vulnerability to stress (Liu et al. 1997; Ladd et al. 2000; Sanchez et al. 2001). A number of animal studies have shown that adverse early life experiences can result in adults that are more susceptible to seizure activity (Frye and Bayon 1999; Edwards et al. 2002; Huang et al. 2002; Lai et al. 2006; Salzberg et al. 2007; Jones et al. 2009). However, many of those studies were based on the analysis of wildtype animals with normal brain and stress system development, and seizure activity was artificially induced in adulthood. In contrast, much less is known about the effect of early life stress on seizure outcomes in genetic models of epilepsy. Seizure activity in the epilepsy (EL) mouse has been shown to be dependent upon maternal care and the presence or absence of the sire during the postnatal period (Orefice and Heinrichs 2008; Leussis and Heinrichs 2009). While the results obtained from the EL mouse studies show that the early life environment can

interact with a genetic predisposition to shape a future seizure phenotype, it is difficult to ascribe the results from these studies to a specific gene or mechanism. The goal of this study was to investigate the role of early life experience on adult behavior and seizure outcome using the *Scn8a*$^{med/+}$ model. Results from this study could highlight the role of a VGSC in the shaping and molding of the adult brain in conjunction with early life experiences. Furthermore, the design of this study will allow us to investigate sex differences following early life manipulation as well as interactions between a VGSC mutation and sex. The early life experience chosen for this set of experiments was a commonly used maternal separation paradigm.

3.3 Methods and Materials

Experimental Subjects

Scn8a$^{med/+}$ mice were purchased from The Jackson Laboratories (Bar Harbor, ME) and maintained on the C3HeB/FeJ background. Genotyping was performed on tail DNA using The Jackson Laboratory protocol. Following the maternal separation paradigm, adult male and female mice, 3-4 months old, were used for all other experiments. Wildtype (WT) littermates were used as controls for all experiments to minimize variation due to differences in genetic background and rearing conditions. Only one male and one female of each genotype from each litter were used in the adult experiments to minimize the impact of variation in maternal care. The experimental mice were randomly chosen from each litter. Mice were group housed after weaning (P21) in ventilated cages under uniform conditions with a 12/12 hour light/dark cycle with lights on at 7 am and

lights off at 7 pm. Mice remained group housed until one week prior to testing, when they were single-housed. Food and water were available *ad libitum*. All testing was done between 8 am and 1 pm to minimize variation due to circadian factors. All experiments were approved by the Emory University Institutional Animal Care and Use Committee (IACUC).

Maternal Separation and Experimental Groups

A total of 47 litters were used for these experiments, divided into two separate cohorts of animals (Cohort 1: MS, n=13 litters; DH, n=12 litters; Cohort 2: MS, n=11 litters; DH, n=11 litters). Litters were randomly assigned to one of two early life 'experiences': handling and maternal separation (MS; n=24 litters) or control daily handling (DH; n=23 litters). All dams were wildtype (WT) C3HeB/FeJ females bred to heterozygous *Scn8a*$^{med/+}$ males. Each dam was used only once for breeding to avoid the possibility that the maternal care of subsequent litters might have been affected by the prior exposure of the dam to the separation protocol. Mice from all litters were toe-clipped on P2 in order to track individual weights. From P2 until P14, pups were separated daily from their dams for 180 minutes (8 am – 11 am, MS) or 15 minutes (8:00 am – 8:15 am, DH). The dam was first removed to a clean cage, and then pups were individually removed from the home cage. Mouse pups were individually handled and weighed. Following the weighing, the DH pups were returned to the home cage and the mother was then placed back into the home cage. The separated MS pups were placed into individual plastic cups and placed in a humidified incubator maintained at 37°C. After 180 minutes, the pups were returned to the home cage, followed by the dam. From P15 until P21, all pups were handled and weighed once daily following the same separation protocol described for the

DH animals. After weaning (P21), mice were handled and weighed once a week through postnatal week 8. Mice were then left undisturbed for a month prior to the initiation of the adult experiments. Mice were weighed one final time at 3 months of age. For the experiments performed during adulthood, the mice were divided into eight groups: 1) Male DH WT, 2) Male DH *Scn8a*$^{med/+}$, 3) Male MS WT, 4) Male MS *Scn8a*$^{med/+}$, 5) Female DH WT, 6) Female DH *Scn8a*$^{med/+}$, 7) Female MS WT, and 8) Female MS *Scn8a*$^{med/+}$. For EEG analysis experiments, nine mice per group were used. For all other adult experiments, 12 mice per group were used. For effects of the MS paradigm, MS groups were compared with DH groups within the same sex. To analyze sex effects, only the DH groups were used: male DH groups were compared with female DH groups to investigate effects of sex on the responses measured without the added factor of MS.

Maternal Behavior

On completion of the analysis of the first cohort of animals, we noticed that the MS group seemed to have improved outcomes when compared to the DH group. Thus, we decided to also measure maternal behavior during the maternal separation paradigm of the second cohort of litters (MS, n=11 litters; DH, n=11 litters). Observations of maternal behavior were performed during the period when the pups were P2-P21, and were conducted three times a day: 1) immediately prior to separation, 2) immediately following return of the dam to the home cage following separation, and 3) one hour after return of the dam to the home cage following separation. Each of the three daily observation periods included three individual observations, spaced two minutes apart, for a total of nine observations per day and 180 total observations for the entire period from P2 until P21. Behavior was scored as either being present or absent during each

observation, and then the percentage of the number of times a behavior was observed out of the 180 total observations was calculated. Maternal behaviors scored included nursing, physical contact with pups, licking pups, sniffing pups, and carrying pups. Non-maternal behaviors scored included eating, drinking, self-grooming, and nest building. Whether or not pups were vocalizing during the observation period was also measured. More than one behavior could have been scored during one observation, such as if the mother was grooming herself while in physical contact with the pups.

Acute Stressor

Mice were stressed as described in Chapter 2 (Section 2.3).

EEG Analysis of Spontaneous Seizure Activity

EEG and EMG electrodes were placed as described in Chapter 2 (Section 2.3). Data was analyzed for the presence of SWDs as previously described. Following 24 h of habituation, continuous EEG and EMG data were collected for a 48-h baseline period. To examine circadian variation, SWDs were grouped into 2-h intervals and averaged within each interval over day 1 and day 2 of the baseline recordings. Following the baseline recording, the seizure response to acute restraint stress was determined by placing animals into modified restraint tubes (50-mL conical plastic tube with a 1.5x1.5 cm^2 hole cut out for EEG headcap placement) for 20 min. Continuous EEG and EMG data were collected during the 20-min restraint period, as well as for the 24-h period following restraint (post-restraint, PR). To examine the response to stress, SWDs were analyzed in 2-h intervals for the 24-h PR period. The first 3-h PR period was also analyzed by further sub-dividing SWD occurrence into 20-min bins.

Flurothyl Seizure Induction

Flurothyl seizure induction was performed as we previously described (Martin et al. 2007; Dutton et al. 2011). Briefly, mice were placed into a clear acrylic chamber and exposed to flurothyl (2,2,2-trifluroethylether) (Sigma) at a rate of 20 µL/min. The latencies to the first myoclonic jerk (MJ), the first generalized tonic-clonic seizure (GTCS), and a GTCS with hindlimb extension were scored. The number of GTCSs experienced prior to the GTCS with hindlimb extension was also counted.

Picrotoxin Seizure Induction

Picrotoxin seizure induction was performed as previously described in Chapter 2 (Section 2.3) with the exception that in this study the mice were not first subjected to restraint stress.

Corticosterone Measurements

Blood was collected from each animal multiple times as follows: First, blood was drawn at 7 pm in order to coincide with the expected peak of circulating corticosterone (CORT) levels. A week following this first blood draw, the effect of an acute stress on the HPA axis stress response and on subsequent evening CORT levels was measured as follows: animals were sampled for a basal CORT level between 10-10:30 am. Immediately following this blood draw, animals were subjected to a 20-min acute restraint stress. Blood was drawn immediately after the restraint stress and then again at 7 pm that evening for a post-stress evening CORT measurement. For all basal CORT measurements, blood was obtained within 1 minute of cage disturbance. Blood was collected from the facial vein into Microvette CB 300 Z tubes (Starstedt) and then

allowed to clot for 1 hour at room temperature. The serum was then separated by centrifugation at 5600 rpm for 15 minutes at 4°C and stored at -80°C until analysis. Serum CORT levels for the diurnal blood draws were assayed using a commercial radioimmunoassay (RIA) kit (MP Biomedicals) according to the manufacturer's instructions.

Open Field Test

The open field apparatus consisted of a square arena (60x60 cm^2) with opaque plexiglas walls (47 cm high). The center zone was designated as a smaller square (30x30 cm^2) with sides 15 cm from the outer walls on all sides. Each mouse was placed along one side of the apparatus and allowed to explore for 15 minutes. Mice were videotaped and behavior was scored using the ANY-maze Video Tracking System (Stoelting Co). Behaviors scored included time spent in the center zone, latency to enter the center zone, total distance travelled, average speed, and total time spent immobile.

Novel Cage Response

The response to novelty was measured as previously described in Chapter 2 (Section 2.3).

Forced Swim Test

The forced swim test was conducted as described in Chapter 2 (Section 2.3).

Statistics

The data are reported as mean ± standard error of the mean (SEM). To analyze effects of early life experience, DH groups were compared to MS groups within a sex. To analyze effects of sex, DH males were compared to DH females to avoid interactions between early life experience and sex. When comparing only two groups of unrelated parametric data, the data were analyzed using the Student t-test. Parametric data sets with two or more groups/factors to be compared were analyzed using two-way analysis of variance (ANOVA) if there was only one measurement per animal or two-way repeated measures ANOVA (rANOVA) if there were multiple data values from a single animal. In experiments with more than three factors, a three-way ANOVA was used. Following the ANOVA analyses, the Tukey pairwise comparison test was used to further distinguish among groups. Nonparametric data were analyzed using the Mann-Whitney Rank Sum test. Dichotomous data were analyzed using the Fisher Exact test. All results were considered statistically significant if $p<0.05$.

3.4 Results

3.4.1 Maternal separation (MS) paradigm unexpectedly increases maternal behavior in C3HeB/FeJ dams

After observing improved outcomes in the first cohort of adult mice that had been subjected to maternal separation we decided to characterize maternal behavior of the second cohort of animals. We unexpectedly found that the wildtype C3HeB/FeJ dams in the maternal separation (MS) paradigm showed increased amounts of maternal care (**Fig. 3.1**A). MS dams nursed their pups more ($t_{(20)}$=-5.079, $p<0.001$), spent more time in physical contact with their pups ($t_{(20)}$=-4.417, $p<0.001$), and licked their pups more often ($t_{(20)}$=-7.326, $p<0.001$) than DH dams. It was observed that when the DH dams were placed back into the home cage with their pups following the 15-minute separation, they were more likely to pick up their pups and move them around the cage, triggering the pups to emit vocalizations. Likewise, we found a higher rate of pup carrying in the DH dams ($t_{(20)}$=2.576, $p<0.05$), and a trend for increased audible vocalizations in the DH pups ($t_{(20)}$=1.782, $p=0.09$). By comparison, the MS dams were more likely to immediately start licking and nursing their pups when returned to the home cage after a 3-hour separation.

3.4.2 MS affects weight gain in both males and females, with stronger effects in males; Genotype affects weight in adult females

After determining that genotype did not affect weight gain during development (birth until postnatal week 8), we combined same-sex *Scn8a*$^{med/+}$ mutants and WT

littermates to determine the effect of the MS paradigm on developmental weight gain. From P14 until weaning, MS males gained significantly more weight than the DH males (**Fig. 3.1**B). Not only was there a significant main effect of time as would be expected ($F_{(19,1425)}$=1756.877, p<0.001), but there was a significant interaction between MS and time ($F_{(19,1425)}$=5.535, p<0.001), indicating that the two groups did not gain weight at the same pace. Post-hoc (Tukey) analysis showed that MS males weighed significantly more than DH males from P14 until P21. For the females, there was also a significant main effect of time ($F_{(19,1387)}$=1870.473, p<0.001) and a significant interaction between MS and time ($F_{(19,1387)}$=3.731, p<0.001). While the significant interaction indicates that the MS females gained weight differently than the DH females overall, none of the specific days were significant in the post-hoc analysis. Analysis of the graphical data (**Fig. 3.1**C) shows that the MS females initially gained weight at a slower pace than the DH females, but then began to gain weight faster than the DH females beginning around P14.

Weekly weight measurements (P28-P56) revealed that MS males consistently weighed more than DH males (**Fig. 3.1**D). In a two-way rANOVA of the weekly weights, there was a trend for a main effect of MS ($F_{(1,75)}$=3.666, p=0.059) and a significant main effect of time ($F_{(4,300)}$=2894.219, p<0.001). The lack of a significant interaction means that the two groups gained weight at about the same rate, but the MS males consistently weighed more than the DH males, likely because of the increased growth rate during the pre-weaning period. There were no differences in weights between MS females and DH females during this period (data not shown).

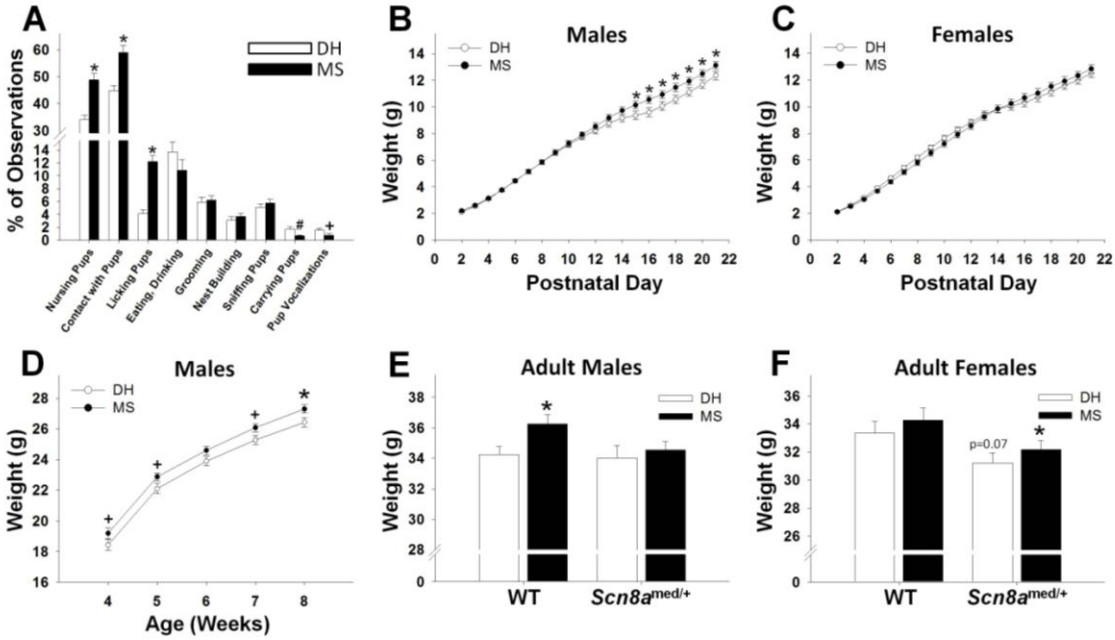

Figure 3.1 Maternal behavior and growth rates in maternal separation paradigm. (A) Wildtype C3HeB/FeJ dams in the maternal separation (MS) group showed increased maternal care as indicated by significantly higher frequencies of nursing, physical contact, and licking as compared to dams in the control daily handling (DH) group. MS dams also carried their pups around less, and their pups were less likely to vocalize. *p<0.001 vs. DH; #p<0.05 vs. DH; +p=0.09 vs. DH. n=11 dams in each group. **(B)** From P14 until P21, MS males (n=39, genotypes combined) gained weight at a faster rate than DH males (n=38). *p<0.05 vs. DH. **(C)** MS females (n=42, genotypes combined) initially gained weight slower than DH females (n=33), but then began to gain weight faster beginning at P14. Two-way rANOVA reports a significant interaction between MS and time ($F_{(19,1387)}$=3.731, p<0.001). **(D)** Analysis of weekly weights showed that MS males consistently weighed more than DH males. *p<0.05 vs. DH; +p=0.06-0.07 vs. DH. **(E)** Once adults, only the WT MS males (n=15) weigh significantly more than the WT DH males (n=21). There are no weight differences due to early life experience in male *Scn8a*$^{med/+}$ mutants. *p<0.05 vs. WT within condition. **(F)** In adult females, early life experience did not affect weights, but *Scn8a*$^{med/+}$ females weighed significantly less than WT female littermates. *p<0.05 vs. WT within condition. Error bars represent SEM.

Interestingly, in the final weight measurement in the adult animals, the genotypes were no longer the same and had to be analyzed separately (**Fig. 3.1**E,F). In males, the increased weight seen originally in the MS animals persisted only in the WT littermates (**Fig. 3.1**E). In *Scn8a*$^{med/+}$ mutants, there were no differences in weight between adult MS

males and adult DH males. Overall, the two-way ANOVA revealed a main effect of MS in the males ($F_{(1,73)}$=4.122, $p<0.05$), but no main effect of genotype nor interaction between MS and genotype. In analysis of adult weight in the females, there was no main effect of MS, but there was a significant main effect of genotype ($F_{(1,71)}$=7.569, $p<0.01$). Post-hoc analysis revealed that *Scn8a*$^{med/+}$ females weighed less than their WT littermates (**Fig. 3.1**F).

3.4.3 Baseline SWD activity and circadian rhythm of SWDs remain unchanged

Our results show that MS did not affect baseline seizure activity, nor the baseline 24-hour rhythm of spontaneous SWD activity in male or female *Scn8a*$^{med/+}$ mutants. Furthermore, there are no sex differences in baseline seizure activity nor 24-hour SWD rhythm (See **Appendix A1**)

3.4.4 MS affects SWD response to acute stress in male *Scn8a*$^{med/+}$ mutants only

We have previously reported that acute restraint stress increases spontaneous SWD activity in the two-hour period immediately following the administration of a 20-minute acute restraint stress in *Scn8a*$^{med/+}$ mutants, but not in WT littermates (Sawyer et al. 2014). As expected in the current study, acute restraint stress did not affect the SWD response in the WT littermates (data not shown), so we have included the analysis of only the *Scn8a*$^{med/+}$ mutants. In examining the immediate spontaneous SWD response to a 20-minute acute restraint stress in the DH males, we found that there is a significant peak in

SWD activity 60-80 minutes following the onset of the restraint stress (**Fig. 3.2**A). Interestingly, in the MS males, the peak of SWD activity following the restraint stress was broader, lasting from 40-100 minutes following the onset of the stressor (**Fig. 3.2**B). In a direct comparison of the DH males with the MS males, it can be seen that the MS males had the largest and broadest response to the acute restraint stress (**Fig. 3.2**C, blue line) compared to the response of the DH males (**Fig. 3.2**C, red line). A three-way

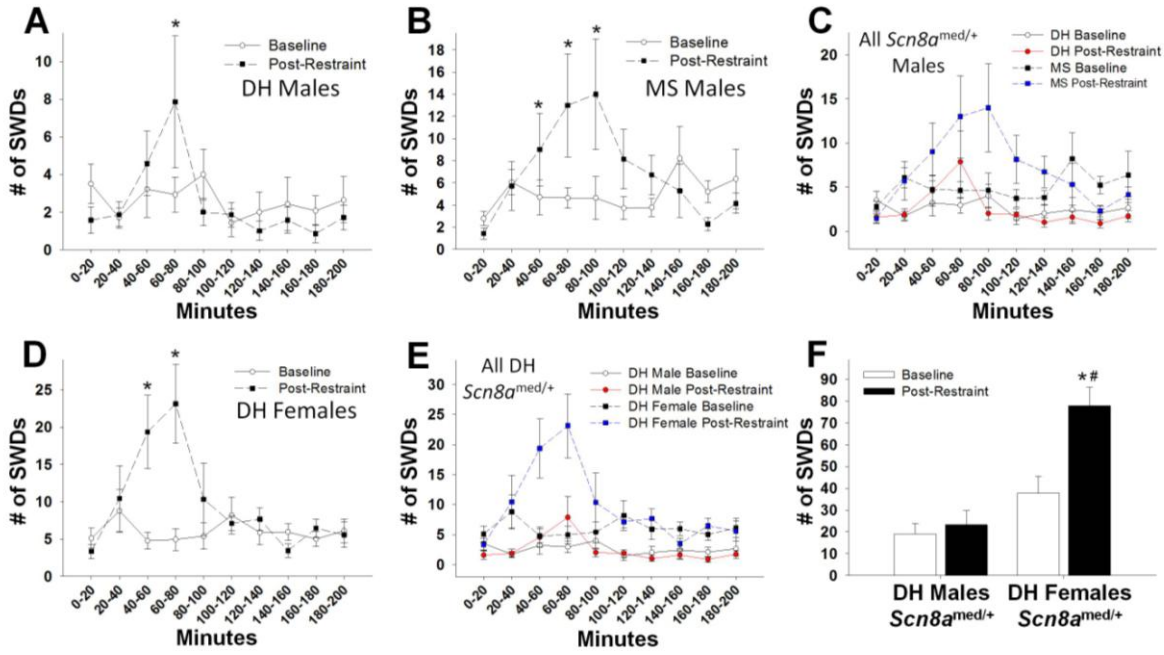

Figure 3.2 Immediate effect of acute restraint stress on spontaneous SWD activity. An acute 20-minute restraint stress (minutes 0-20) increased SWD activity for a short period in both (A) DH males and in (B) MS males. (C) In a direct comparison between DH and MS males, there was a trend for an interaction between MS and restraint stress, with the MS males (blue line) having a larger and broader SWD response to the stress than the DH males (red line). (D) In response to restraint stress, DH females also displayed a significant increase in SWD activity. (E) In a comparison of DH males and DH females, the females (blue line) have a larger and broader increase in SWD in response to the stressor than the males (red line). (F) The sex difference is also apparent in a comparison of the two-hour period post-restraint with the same two-hour period at baseline. n=9 all groups; *p<0.05 vs. Baseline; #p<0.05 vs. Males within post-restraint. Error bars represent SEM.

rANOVA comparing the factors of time, restraint stress, and MS revealed a main effect of time ($F_{(9,144)}$=3.534, p<0.01) and a significant interaction between time and restraint stress ($F_{(9,144)}$=3.989, p<0.001); however, the interaction between MS and restraint stress was not as strong ($F_{(1,16)}$=3.495, p=0.08). This indicates that while restraint stress was certainly increasing SWD activity in all animals, there was only a trend for MS affecting the SWD response to the acute restraint stress.

3.4.5 DH Females have a larger SWD response to acute stress than DH males

There were no indications that MS affected the female SWD response to an acute stressor in any way (data not shown). The response of the MS females looked very similar to the response of the DH females which is shown in Figure 2D. Acute restraint stress significantly increased SWD seizure activity in all female *Scn8a*$^{med/+}$ mutants from 40-80 minutes following the onset of the restraint stress (**Fig. 3.2**D). However, in a comparison between males and females, the females had a much larger and broader SWD response than males to the acute restraint stress (**Fig. 3.2**E, blue line). A three-way rANOVA revealed a significant main effect of sex ($F_{(1,16)}$=12.149, p<0.01), a significant main effect of restraint stress ($F_{(1,16)}$=11.230, p<0.01), and a significant main effect of time ($F_{(9,144)}$=4.604, p<0.001). There was also a significant interaction between restraint stress and time ($F_{(9,144)}$=5.566, p<0.001) and a significant interaction between restraint stress and sex ($F_{(1,16)}$=14.229, p<0.01), indicating that the two sexes were not responding to the restraint stress in the same way. **Figure 3.2**F shows the huge sex difference in response to an acute restraint stress by showing the total number of seizures in the two-

hour period following the restraint stress as compared to the same two-hour period during baseline. A two-way rANOVA of these values revealed a main effect of sex ($F_{(1,16)}$=16.224, p<0.001), a main effect of restraint stress ($F_{(1,16)}$=30.433, p<0.001), and a significant interaction between sex and restraint stress ($F_{(1,16)}$=19.944, p<0.001). While the males showed a slight increase in SWD activity summed over the 2-hour period post-restraint, the difference in the females was much larger (**Fig. 3.2**F).

3.4.6 Long-term effect of restraint stress on SWD activity remains unchanged

MS does not affect the long-term effects of restraint stress on SWD activity. All groups of *Scn8a*$^{med/+}$ mutants showed a broadened peak of seizure activity in the evening following the morning exposure to the restraint stress (see **Figure A2.1** in **Appendix A2**). There were also no sex differences in the long-term effects of stress on SWD activity (See **Appendix A2**).

3.4.7 MS improves flurothyl-induced seizure outcome in *Scn8a*$^{med/+}$ females only

All flurothyl results are listed in **Appendix A3**. There were many significant genotype effects, which was as expected since *Scn8a*$^{med/+}$ is known to have higher thresholds to induced seizures when compared to WT littermates. However, there was only one significant effect of maternal separation; MS *Scn8a*$^{med/+}$ females had a much longer average latency to the more severe GTCS (running/bouncing and progression to

hindlimb extension) than DH *Scn8a*$^{med/+}$ females (see **Table A3.1** in **Appendix A3**). No effects of MS were observed in the males. We also only detected one sex difference. While female *Scn8a*$^{med/+}$ mutants have a significantly higher threshold to the flurothyl-induced generalized seizure than their WT littermates, the increase in threshold in much larger in the male *Scn8a*$^{med/+}$ mutants. The latencies between male WT littermates and female WT littermates were comparable, suggesting a sex-genotype interaction where sex is only playing a role in the latencies of the *Scn8a*$^{med/+}$ mutants.

3.4.8 MS improves picrotoxin-induced seizure outcome in *Scn8a*$^{med/+}$ males only

To further examine the effect of MS on induced seizure activity, we injected mice with picrotoxin (5 mg/kg), a potent GABA antagonist. In analyzing the latency to a Stage 2 seizure (atypical clonic seizure), we found a main effect of genotype ($F_{(1,38)}$=4.209, $p<0.05$). While there was no effect of MS and no genotype-MS interaction, the post-hoc analysis showed that only the MS *Scn8a*$^{med/+}$ males had a significant increase in latency to the Stage 2 seizure (**Fig. 3.3**A). Analysis of the latency to a Stage 3 seizure (bilateral forelimb clonus) showed a main effect of genotype ($F_{(1,38)}$=8.407, $p<0.01$), a main effect of MS ($F_{(1,38)}$=4.776, $p<0.05$), and a significant interaction between genotype and MS ($F_{(1,38)}$=4.354, $p<0.05$). Post-hoc analysis revealed that again only the MS *Scn8a*$^{med/+}$ males had a significant increase in latency to the Stage 3 seizure (**Fig. 3.3**B). Analysis of the Stage 4 seizure (GTCS) showed a only main effect of genotype ($F_{(1,38)}$=11.777, $p<0.001$). Post-hoc analysis again revealed that only the MS *Scn8a*$^{med/+}$ males had a significant increase in latency (**Fig. 3.3**C). Finally, we analyzed seizure severity by using

a modified Racine scale. A two-way ANOVA revealed a main effect of genotype ($F_{(1,38)}$=15.537, p<0.001) and a main effect of MS ($F_{(1,38)}$=4.077, p=0.05). As expected in this line, both groups of *Scn8a*$^{med/+}$ mutants showed decreased seizure severity, while the MS *Scn8a*$^{med/+}$ mutants showed a trend for reduced severity as compared to DH *Scn8a*$^{med/+}$ mutants (**Fig. 3.3**D). Overall, the early life experiences of the MS *Scn8a*$^{med/+}$ mutants resulted in an improved seizure outcome in response to picrotoxin.

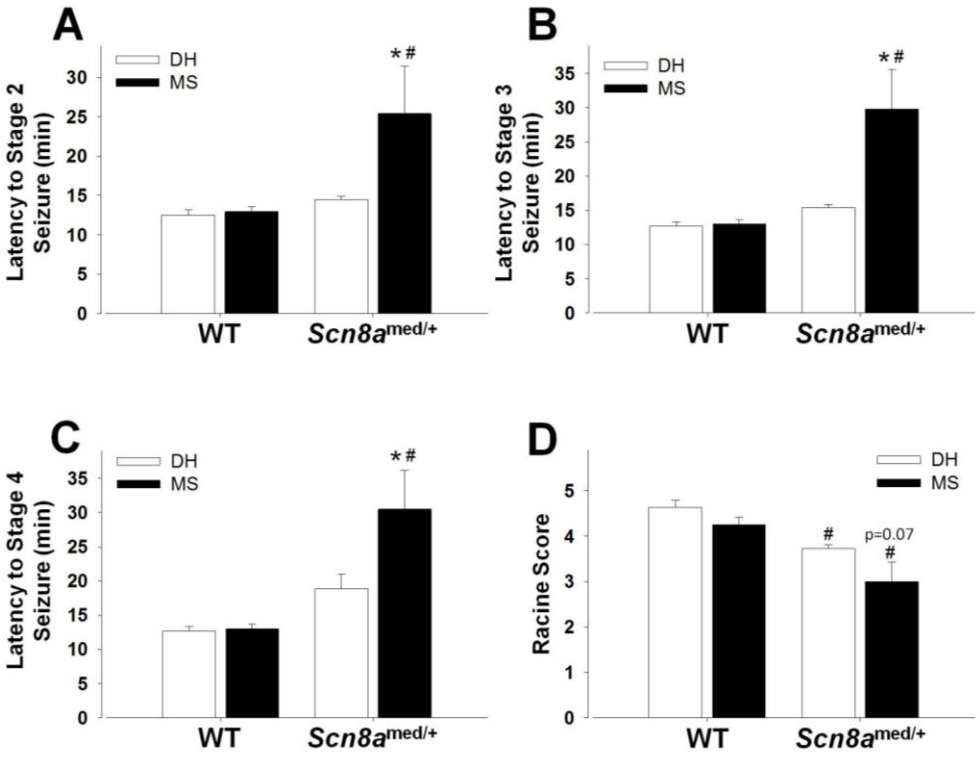

Figure 3.3 MS improves picrotoxin-induced seizure outcome in *Scn8a*$^{med/+}$ males only. (A-C) In response to picrotoxin (5 mg/kg), only the MS *Scn8a*$^{med/+}$ mutants showed an increased latency to a Stage 2 seizure (atypical clonic seizure) **(A)**, a Stage 3 seizure (bilateral forelimb clonus) **(B)** and a Stage 4 seizure (GTCS) **(C)**. **(D)** In a measure of seizure severity, there was a trend for MS to reduce the severity of seizure activity in the *Scn8a*$^{med/+}$ mutants (p=0.07). *p<0.05 vs. DH within genotype. #p<0.05 vs. WT within condition. n=12 all groups. Error bars represent SEM.

3.4.9 DH females are more resistant to picrotoxin-induced seizures than DH males

We compared the DH males and DH females to determine the effects of sex on seizure outcome following injection with picrotoxin. Analysis of the latency to the Stage 2 seizure revealed a main effect of sex ($F_{(1,33)}$=4.809, $p<0.05$) and a main effect of genotype ($F_{(1,33)}$=4.420, $p<0.05$). Post-hoc analysis revealed that only the *Scn8a*$^{med/+}$ females had a significantly increased latency to a Stage 2 seizure (**Fig. 3.4**A). Results were similar for the latency to the Stage 3 seizure. There was a main effect of sex ($F_{(1,33)}$=6.709, $p<0.05$) and a main effect of genotype ($F_{(1,33)}$=5.442, $p<0.05$). Post-hoc analysis showed that, again, only the *Scn8a*$^{med/+}$ females had a significantly increased latency (**Fig. 3.4**B). Analysis of the latency to a Stage 4 seizure revealed a main effect of sex ($F_{(1,33)}$=13.538, $p<0.001$), a main effect of genotype ($F_{(1,33)}$=19.980, $p<0.001$), and a significant interaction between sex and genotype ($F_{(1,33)}$=6.954, $p<0.05$). Again, post-hoc analysis revealed that only the *Scn8a*$^{med/+}$ females had a significant increase in their latency to a Stage 4 seizure (**Fig. 3.4**C). It is interesting to note that in these earlier stages of the picrotoxin-induced seizure progression, only the *Scn8a*$^{med/+}$ females showed an improvement in seizure outcome, suggesting that there is an interplay at work between genotype and sex (**Fig. 3.4**A-C).

However, once the picrotoxin-induced seizure had progressed to its final stage, the Stage 5 seizure (GTCS with hindlimb extension), females of both genotypes show an increased resistance to seizure activity (**Fig. 3.4**D). Two-way ANOVA analysis revealed a main effect of sex ($F_{(1,33)}$=23.565, $p<0.001$) and a main effect of genotype ($F_{(1,33)}$=15.598, $p<0.001$). Post-hoc analysis showed that females of both genotypes had

an increased latency to the Stage 5 seizure (**Fig. 3.4**D). Upon further examination, it was observed that fewer females made the progression to the Stage 5 seizure. Only 25% of the WT females and none of the *Scn8a*$^{med/+}$ females progressed to the GTCS with hindlimb extension, while 100% of the WT males and 44.4% of the *Scn8a*$^{med/+}$ males progressed to this final stage (**Fig. 3.4**E). Overall, it appears that females are more resistant to picrotoxin-induced seizures, with the *Scn8a*$^{med/+}$ females taking the longest time to progress through the various seizure stages.

There were no differences in measures of seizure severity between males and females (data not shown; Full picrotoxin data set in **Appendix A4**).

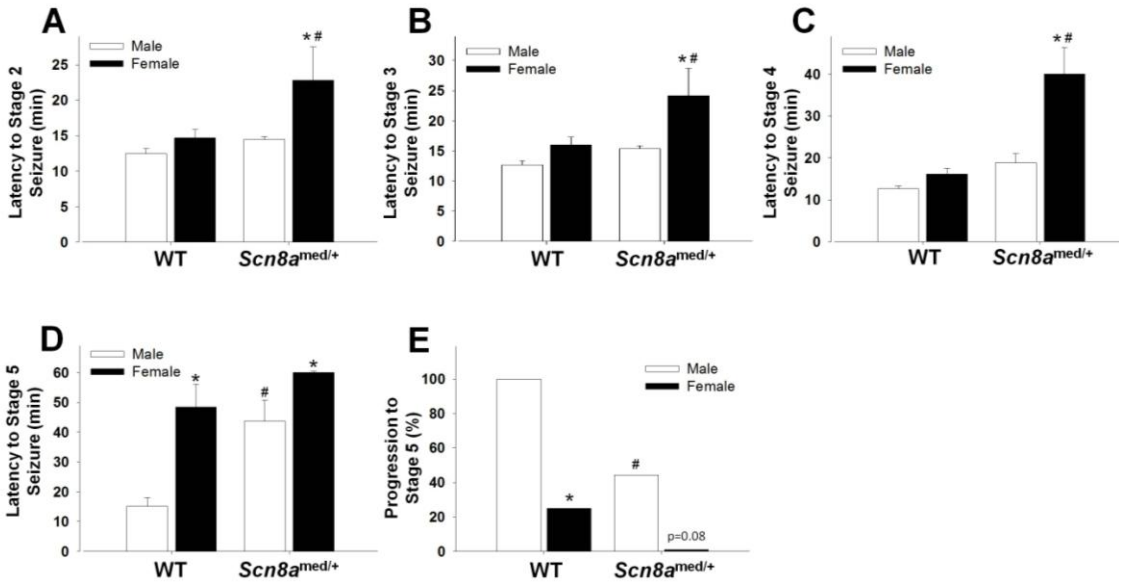

Figure 3.4 *Scn8a*$^{med/+}$ Females have higher latencies to picrotoxin-induced seizures than *Scn8a*$^{med/+}$ males. (A-C) In response to picrotoxin (5 mg/kg), only the *Scn8a*$^{med/+}$ females showed an increased latency to a Stage 2 seizure (atypical clonic seizure) **(A)**, a Stage 3 seizure (bilateral forelimb clonus) **(B)** and a Stage 4 seizure (GTCS) **(C)**. **(D)** Females of both genotypes had a higher latency to the Stage 5 seizure (GTCS with hindlimb extension). **(E)** While fewer *Scn8a*$^{med/+}$ mutants progressed to a Stage 5 seizure overall, females of both genotypes were less likely to progress to this stage compared to males. *p<0.05 vs. Male within genotype. #p<0.05 vs. WT within sex. n=12 all groups. Error bars represent SEM.

3.4.10 MS reduces the CORT response to an acute stressor in females only

There were no genotype differences or MS effects on basal CORT levels or the 7 PM CORT levels following exposure to an acute restraint stress in the morning. Furthermore, as expected based on previous studies demonstrating sex differences in the functioning of the HPA axis (Kudielka and Kirschbaum 2005), females had significantly higher levels of CORT than males across all measures (data not shown; see **Appendix A5**). The only other noteworthy results from these CORT experiments came from the analysis of the CORT response to an acute 20-minute restraint stress. Interestingly, the analysis of the males revealed a main effect of genotype ($F_{(1,38)}=5.012$, $p<0.05$), with *Scn8a*$^{med/+}$ mutants having an higher CORT response to the stressor than their WT littermates (**Fig. 3.5**A). We had previously reported no genotype difference between male *Scn8a*$^{med/+}$ mutants and WT littermates in the CORT response to an acute stressor (Sawyer et al. 2014). However, post-hoc analysis of the results from the current study show that the difference appears to be largely in the male MS *Scn8a*$^{med/+}$ mutants, suggesting that MS is again interacting with the genotype to change the phenotype. In females, we found a strong trend for a main effect of MS ($F_{(1,38)}=4.007$, $p=0.052$), with MS females having a reduced CORT response to the acute stressor (**Fig. 3.5**B).

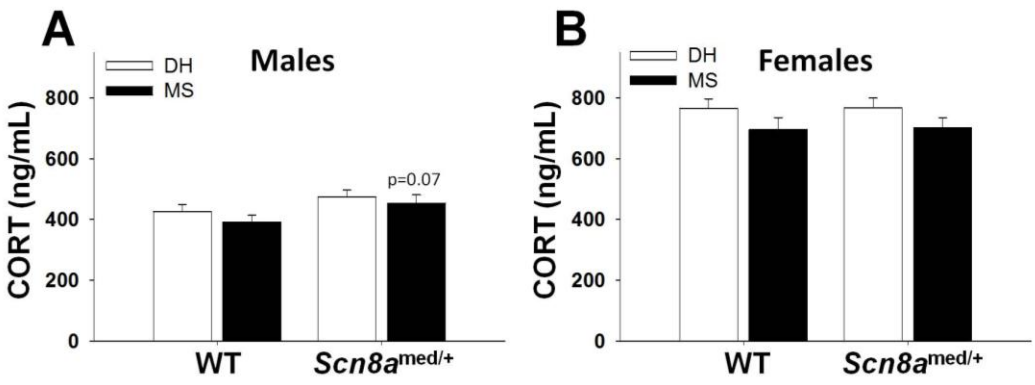

Figure 3.5 Corticosterone (CORT) Response to Acute Restraint Stress. (A) In males, there was a significant main effect of genotype ($F_{(1,38)}$=5.012, p<0.05) in the CORT response to an acute 20-minute restraint stress. MS *Scn8a*$^{med/+}$ males showed a trend towards higher CORT levels than MS WT littermates. **(B)** In females, there was a trend towards a main effect of maternal separation ($F_{(1,38)}$=4.007, p=0.052), with MS females having a reduced CORT response to an acute 20-minute restraint stress. n=12 all groups. Error bars represent SEM.

3.4.11 MS affects males and females differently in open field measures

To test for changes in locomotor and anxiety-like behaviors, we use the open field paradigm. Interestingly, MS seemed to affect males and females differently. In the first measure tested, total distance traveled, we found that male MS animals traveled less distance than the DH animals (**Fig. 3.6**A). While there was a main effect of MS ($F_{(1,38)}$=5.189, p<0.05), post-hoc analysis showed that there was only a strong trend (p=0.056) for reduced distance traveled by MS *Scn8a*$^{med/+}$ males as compared to DH *Scn8a*$^{med/+}$ males. In females, we saw that the MS animals traveled a greater distance than the DH animals (**Fig. 3.6**B). Again there was a main effect of MS ($F_{(1,38)}$=4.705, p<0.05), and while there were no significant post-hoc results, the data show that on average, the

MS female mice traveled more than the DH females. In a comparison of sexes within the DH group only, it is apparent that females travel less than males (**Fig. 3.6**C). Interestingly, since MS was causing males to travel a decreased distance and females to travel an increased distance, the ultimate effect of the MS was to reduce the sex differences that existed in the DH animals.

Analysis of the time spent immobile tells a similar story. In males, MS animals spent more time immobile than DH animals (**Fig. 3.6**D). A two-way ANOVA showed a main effect of MS ($F_{(1,38)}$=6.416, $p<0.05$), while post-hoc analysis showed that the MS *Scn8a*$^{med/+}$ males spent more time immobile than the DH *Scn8a*$^{med/+}$ males. In females, MS animals spent less time immobile than DH animals (**Fig. 3.6**E). Two-way ANOVA analysis showed a trend for a main effect of MS ($F_{(1,38)}$=3.721, $p=0.061$). A sex analysis of only DH animals shows that, on average, females spend more time immobile than males (**Fig. 3.6**F). There was a significant main effect of sex ($F_{(1,37)}$=9.168, $p<0.01$), with females of both genotypes spending more immobile than their male littermates. Similarly to the distance traveled results, MS is causing males to spend more time immobile and females to spend less time immobile, narrowing the sex difference that existed in the DH animals.

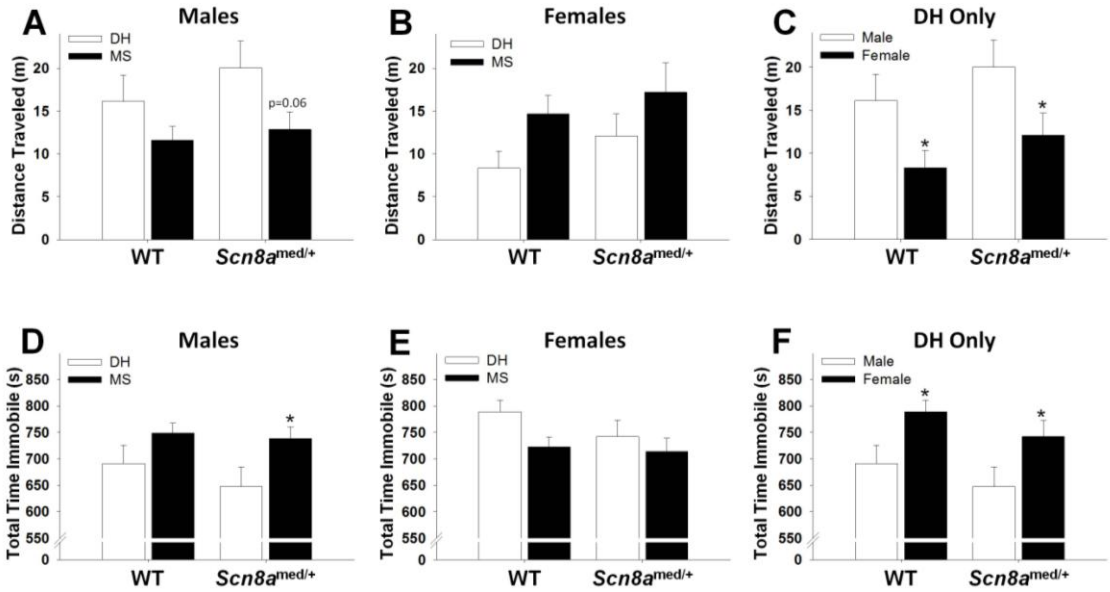

Figure 3.6 MS Affects Males and Females Differently in Open Field Measures. (A) In males, MS animals traveled less distance, with MS *Scn8a*$^{med/+}$ animals showing a stronger trend towards less travel. **(B)** In females, MS animals traveled a farther distance. While there were no significant post-hoc results, there was a main effect of maternal separation ($F_{(1,38)}$=4.705, p<0.05). **(C)** In a direct comparison of DH males and females, it is revealed that regardless of genotype, females traveled less distance than males. *p<0.05 vs. Male within genotype. **(D)** In males, MS animals spent more time immobile. *p<0.05 vs. DH within genotype. **(E)** In females, MS animals spent less time immobile. While no post-hoc results were significant, there was a trend for a main effect of maternal separation ($F_{(1,38)}$=3.721, p=0.06). **(F)** In a direct comparison of DH males and females, females spent more time immobile than males. *p<0.05 vs. Male within genotype. n= 12 all groups. Error bars represent SEM.

Because the C3HeB/FeJ strain does not explore as much in the open field as do other strains of mice (personal observation), we were unable to use the center measures to test anxiety-like behaviors. Mice very rarely ventured into the center of the open field, preferring to stay next to the walls of the apparatus at all times. Thus, while it could be argued that traveling less and spending more time immobile could be attributed to an increase in anxiety-like behavior, it could just as easily be due to alterations in locomotor

ability. We believe that this set of experiments, however, shows that MS is having an effect on open field measures, albeit a different effect depending on the sex of the animal.

3.4.12 MS mildly affects novel cage and forced swim behavior in females

All results from the novel cage test and forced swim test are shown in **Table 3.1** and **Table 3.2**. **Table 3.1** shows the significant main effects and significant interactions from the ANOVA. **Table 3.2** shows the means and the significant post-hoc results. We have previously published that male *Scn8a*$^{med/+}$ mutants have increased anxiety-like behavior in a novel cage paradigm, characterized by increased grooming and decreased exploratory behaviors (Sawyer et al. 2014). In the present study, we replicated these effects in the males by showing that the male *Scn8a*$^{med/+}$ mutants (MS and DH) spend more time immobile, rear less, and groom more. Interestingly, while female *Scn8a*$^{med/+}$ mutants also rear less and groom more, MS causes a significant reduction in female grooming in the *Scn8a*$^{med/+}$ mutants only and not the WT littermates. MS also caused female WT animals to engage in more stretch-attends, a type of exploratory behavior. It appears that in females, but not males, MS is having a mild anti-anxiogenic effect in the novel cage environment.

Table 3.1 ANOVA Results from Novel Cage and Forced Swim Behavioral Tasks

	Significant Main Effects	Significant Interactions	Summary of Results
MALES ONLY:			
NC: Time Immobile (s)	Genotype: $F_{(1,38)}$=3.366, p=0.074	N/A	*Scn8a*$^{med/+}$ are more immobile
NC: Number of Rears	Genotype: $F_{(1,38)}$=4.876, p<0.05	N/A	*Scn8a*$^{med/+}$ rear less
NC: Time Grooming (s)	Genotype: $F_{(1,38)}$=4.478, p<0.05	N/A	*Scn8a*$^{med/+}$ groom more
NC: # of Stretch-Attends	N/A	N/A	N/A
FST: Total Time Floating (s)	Genotype: $F_{(1,35)}$=7.290, p<0.05	N/A	*Scn8a*$^{med/+}$ float more
FST: Latency to Float (s)	N/A	N/A	N/A
FEMALES ONLY:			
NC: Time Immobile (s)	N/A	N/A	N/A
NC: Number of Rears	Genotype: $F_{(1,38)}$=5.100, p<0.05	N/A	*Scn8a*$^{med/+}$ rear less
NC: Time Grooming (s)	MS: $F_{(1,38)}$=5.501, p<0.05	Genotype & MS: $F_{(1,38)}$=7.451, p<0.01	*Scn8a*$^{med/+}$ groom more MS causes *Scn8a*$^{med/+}$ to groom less
NC: # of Stretch-Attends	Genotype: $F_{(1,38)}$=5.240, p<0.05	Genotype & MS: $F_{(1,38)}$=5.467, p<0.05	MS increases SAs in WT only
FST: Total Time Floating (s)	MS: $F_{(1,37)}$=3.602, p=0.066	N/A	MS increases floating
FST: Latency to Float (s)	MS: $F_{(1,38)}$=5.933, p<0.05	N/A	MS increases latency to float
DH ONLY (GENDER COMPARISONS):			
NC: Time Immobile (s)	N/A	Gender & Genotype: $F_{(1,37)}$=5.473, p<0.05	In WTs, females are more immobile In Hets, females are less immobile
NC: Number of Rears	N/A	Gender & Genotype: $F_{(1,37)}$=9.804, p<0.01	In WTs, females rear less In Hets, females rear more
NC: Time Grooming (s)	Genotype: $F_{(1,37)}$=5.224, p<0.05	N/A	*Scn8a*$^{med/+}$ groom more
NC: # of Stretch-Attends	N/A	N/A	N/A
FST: Total Time Floating (s)	Genotype: $F_{(1,35)}$=6.151, p<0.05	N/A	*Scn8a*$^{med/+}$ float more
FST: Latency to Float (s)	Gender: $F_{(1,37)}$=5.154, p<0.05	N/A	Females have a shorter latency

Table 3.1 shows the significant ANOVA results from the novel cage (NC) and forced swim test (FST) behavioral paradigms, divided into significant main effects and significant interactions, along with a summary of the major findings. We independently analyzed all males together, all females together, and all DH animals together. n=12 all groups.

While we previously reported that there were no genotype differences in the response to the forced swim test between male *Scn8a*$^{med/+}$ mutants and WT littermates (Sawyer et al. 2014), we found that in the current study, male *Scn8a*$^{med/+}$ mutants float more than their WT littermates. However, as with the novel cage paradigm, MS did not seem to affect male behavior. Females, in contrast, did show changes in their forced swim behaviors after undergoing the MS paradigm. The results are seemingly contradictory as MS increases the total amount of time spent floating in the females while it also increases the latency to the first floating episode. While the first result seems to

Table 3.2 Means (±SEM) of Data Obtained from Novel Cage and Forced Swim Behavioral Tasks

	DH WT	DH *Scn8a*$^{med/+}$	MS WT	MS *Scn8a*$^{med/+}$
MALES:				
NC: Time Immobile (s)	718.9 (± 21.1)	754.6 (± 17.0)	685.2 (± 19.3)	734.4 (± 30.8)
NC: Number of Rears	76.3 (± 9.6)	55.4 (± 8.4)	89.3 (± 6.6)	66.5 (± 13.0)
NC: Time Grooming (s)	12.7 (± 1.3)	16.4 (± 2.7)	9.8 (± 2.1)	14.9 (± 2.2)
NC: # of Stretch-Attends	14.9 (± 1.9)	15.5 (± 2.4)	12.4 (± 2.1)	12.0 (± 1.7)
FST: Total Time Floating (s)	41.1 (± 12.3)	*114.4 (± 30.1)	34.9 (± 11.0)	+58.8 (± 8.7)
FST: Latency to Float (s)	115.5 (± 21.4)	126.0 (± 20.4)	136.3 (± 29.5)	156.7 (± 13.1)
FEMALES:				
NC: Time Immobile (s)	757.0 (± 18.8)	*#693.8 (± 26.2)	686.6 (± 29.9)	706.8 (± 24.8)
NC: Number of Rears	53.7 (± 8.4)	*#93.4 (± 11.8)	75.8 (± 10.1)	82.8 (± 10.6)
NC: Time Grooming (s)	14.0 (± 1.6)	*19.6 (± 2.4)	14.7 (± 2.1)	+10.0 (±1.4)
NC: # of Stretch-Attends	12.1 (±1.5)	12.2 (±3.0)	+20.1 (± 1.8)	*10.6 (± 1.6)
FST: Total Time Floating (s)	47.8 (± 15.5)	67.7 (± 9.5)	111.9 (± 31.8)	92.1 (± 24.9)
FST: Latency to Float (s)	86.7 (± 13.2)	#74.0 (± 13.7)	128.8 (± 19.9)	118.7 (± 21.4)

Table 3.2 shows the means (±SEM) from the novel cage (NC) and forced swim test (FST) behavioral paradigms by group. Post-hoc analysis results are shown by the following symbols: *p<0.05 Significant genotype difference (WT vs. *Scn8a*$^{med/+}$ within condition and sex); #p<0.05 Significant sex difference (male vs. female within DH); +p<0.05 Significant MS effect (DH vs. MS within the same genotype and sex). n=12 all groups.

indicate an increase in depressive-like behavior by the MS females, the second result suggests an increase in latency to the depressive-like behavior.

The sex comparisons of the DH animals led to some surprising results. More specifically, in the novel cage experiment, analysis of the total time spent immobile and the number of rears yielded a significant interaction between genotype and sex, where the differences between males and females depended on the genotype. When comparing WT animals, females are more immobile and rear less, both indicative of decreased exploratory behavior. In contrast, when comparing *Scn8a*$^{med/+}$ animals, females are less immobile and rear more, both indicative of increased exploratory behavior. There was

also a sex difference in the forced swim test, with females of both genotypes showing a shorter latency to floating than their male counterparts.

3.5 Discussion

Our findings support the hypothesis that early life experience (in particular, maternal care) can affect adult behavior and seizure outcome. While we initially designed the experiment to study the effects of early life stress on vulnerability to seizures by using the maternal separation paradigm, we observed that maternal separation actually led to increased maternal care by the dams. The maternal separation paradigm was originally designed to be used with rats, and rat pups having undergone this early period of separation from their dams show long-lasting changes in HPA axis function and altered behavior as adults (Caldji et al. 1998; Kalinichev et al. 2002; Macri et al. 2004). Notably, in rats, daily separation of pups from the dam results in decreased maternal care, and decreased maternal care was linked with stress reactivity in the adult animals (Liu et al. 1997). The use of maternal separation in mice is a relatively new paradigm. It appears that the same 3-hour-per-day period of separation from P1 or P2 until P14 in mice results in either increased maternal care or paradoxical results (Millstein and Holmes 2007; Own and Patel 2013). While the maternal separation paradigm has now been tested in several strains of mice, the present study is the first to use the C3HeB/FeJ strain. It appears that in this strain, the maternal separation paradigm results in increased levels of maternal care. Thus, we have interpreted our results as indicative of an altered early life experience and possibly even some early life enrichment rather than as a result of a stress-inducing paradigm.

Our study design resulted in three possible main effects: 1) an effect of the maternal separation (MS) paradigm, 2) an effect of genotype, and 3) an effect of sex. Interactions between these factors were also considered. For simplicity, we have chosen to summarize the main findings within each of the three factors before discussing the complex interactions that appear to be at play within this study.

3.5.1 Effects of MS

Beginning after P14, all MS animals gained more weight than their DH counterparts. In adult males, only the MS WT animals continued to weigh more than the DH WT animals; the effect in the *Scn8a*$^{med/+}$ mutants was lost. While MS appeared to be anti-convulsant in *Scn8a*$^{med/+}$ mutants of both sexes by increasing the threshold to chemically induced seizures (increased flurothyl threshold in *Scn8a*$^{med/+}$ females and increased picrotoxin thresholds in *Scn8a*$^{med/+}$ males), MS also increased spontaneous SWD activity following an acute stress in male *Scn8a*$^{med/+}$ mutants. However, while this immediate response to an acute stressor was larger in MS male *Scn8a*$^{med/+}$ mutants, there were no MS effects apparent in either the basal circadian rhythm of SWDs or the circadian rhythm shift of SWDs caused by acute stress. Interestingly, MS only notably affected induced seizure thresholds in the *Scn8a*$^{med/+}$ mutants, but not in the WT littermates, suggesting an interaction between the MS paradigm and genotype, which will be discussed in more detail shortly. MS had minimal effects on adult HPA axis function, only reducing the CORT response to an acute stressor in females. MS also had opposite effects on open field behavior depending on the sex. While MS males showed decreased

locomotion and exploration, MS females demonstrated more of these traits. Interestingly, MS served to decrease apparent sex differences observed in the DH animals, suggesting that the MS paradigm somehow normalized sex effects on behavior. Furthermore, MS increased exploratory behavior and reduced anxiety behavior in females as measured by the novel cage paradigm, yet it also had paradoxical effects on the forced swim behavior of the females.

3.5.2 Effects of Genotype

We replicated many of our previous findings of the effects of the *Scn8a*$^{med/+}$ mutation on behavior and seizure outcome, including the circadian rhythm of SWD activity seen in *Scn8a*$^{med/+}$ mutants and the altered evening rhythm of SWD activity following an acute stressor in the morning. We also replicated our previous findings of increased anxiety-like behaviors in the *Scn8a*$^{med/+}$ mutants as well as increased thresholds to chemically-induced seizures. Novel genotype findings of this study include the result that female *Scn8a*$^{med/+}$ mutants weigh significantly less than their WT littermates, suggesting a possible role for *Scn8a* in weight management in females. We also found that the male *Scn8a*$^{med/+}$ mutants in this study had a slightly higher CORT response to an acute stressor, in contrast to our previous findings of no HPA axis differences due to the *Scn8a*$^{med/+}$ mutation (Sawyer et al. 2014). This seeming disparity could be due to an effect of the early life experience as even the DH controls were subjected to a brief daily handling, something not done in our previous study. Similarly, we found that the male *Scn8a*$^{med/+}$ mutants in this study floated more in the forced swim test, in contrast to earlier

results. It is possible that the *Scn8a*$^{med/+}$ mutation may mediate or interact with neural changes arising from an altered early life experience, so that some genotype differences are only apparent depending on the early life experience as discussed in further detail in a later section.

3.5.3 Effects of Sex

Perhaps not surprisingly due to the neuroactive effects of sex hormones, we discovered many sex differences. To keep an already complicated analysis from becoming unwieldy, we chose to compare males and females only within the control group, the DH animals. To examine sex effects in the MS group would have introduced too many confounding factors and made interpretation difficult at best. In summary, we found that female *Scn8a*$^{med/+}$ mutants had a stronger SWD response to an acute stress, yet they also had much higher latencies to picrotoxin-induced seizures. In fact, fewer females progressed to a Stage 5 seizure than did males. The females also had higher CORT values in all measures tested, an expected result as it is well established that females have higher CORT levels than males (Bailey and Silver 2013). We also found that females are less active in the open field than males, as indicated by increased immobility and decreased distance traveled. Interestingly, our results in the novel cage paradigm suggest sex differences that are co-dependent on genotype. In WT animals, females were more immobile and reared less, while in the mutants, females were less immobile and reared more. This suggests an interaction between the *Scn8a* mutation and sex, discussed further in a later section.

3.5.4 MS-Sex Interactions

Sex differences in response to early life experiences appear to be a consistent finding within the literature. It is known that early life experiences can shape and alter the responsiveness of the HPA axis in adulthood (Liu et al. 1997). It is also well-known that HPA axis function differs in males and females (Bailey and Silver 2013). Therefore, it is not surprising to discover that in measures of neuroendocrine function, sex is a significant modulator of early life experience on adult stress reactivity (DeSantis et al. 2011). This alteration in stress reactivity can in turn affect seizure activity. For example, in other studies looking at the effects of MS on seizure outcome, results were dependent on the sex of the animals (Edwards et al. 2002; Salzberg et al. 2007). In the present study, MS improved the induced-seizure outcome of both sexes, but this effect was seen with different chemiconvulsants. MS males showed increased latencies to picrotoxin-induced seizures, while MS females showed increased latencies to flurothyl-induced seizures. While both picrotoxin and flurothyl involve inhibition of GABAergic pathways, flurothyl also involves other as yet determined pathways (Velisek 2006). Metabolites of female hormones, such as allopregnanolone derived from progesterone, act as positive modulators of GABA receptors (Morrow 2007), which could explain why females have higher thresholds in general to picrotoxin when compared to males. Since control females are already showing increased thresholds to picrotoxin, our study design may have prevented us from seeing a further increase in seizure threshold in females as a result of the MS paradigm. The MS effect in females was therefore only apparent in another paradigm (flurothyl) which also involved non-GABAergic pathways.

It is also likely that since the current study was more a model of early life enrichment than early life stress, that effect sizes due to our MS paradigm were small. For example, we only saw an effect of MS on CORT levels in females, but not in males. This could be accounted for by the fact that effects of early life experience are typically larger in females than in males (Welberg et al. 2006). The effect we saw in our females was not very large, which suggests that any effects in the male were not necessarily absent, but could have been below the level of detection by the design of our study.

Finally, we noticed an interaction between MS and sex in our behavioral outcomes. In the open field paradigm, MS affected males and females in opposite ways, making the males less active and the females more active. Interestingly, in the control (DH) animals, males demonstrated much higher levels of activity in the open field, traveling a greater distance and spending less time immobile than females. The MS paradigm effectively removed this sex difference by making the overall responses of the two sexes more similar. An emerging theme in the study of early life experience is sex-specific epigenetic effects. In a study by Kundakovic et al. (2013), male C57BL/6J mice exposed to a maternal separation paradigm showed increased hyperactivity in behavioral tasks, while the females did not; furthermore, the mice also showed sex-specific gene expression and epigenetic variation. While the male mice in our study showed a decrease in activity in the open field, this is perhaps not surprising given the emerging knowledge about strain-specific effects in response to early life experience (Kundakovic et al. 2013).

3.5.5 MS-Genotype Interactions

While in the induced-seizure experiments there was a main overall effect of maternal separation, the MS *Scn8a*$^{med/+}$ mutants showed a much higher increase in seizure threshold than their MS WT littermates, suggesting an interaction between MS and genotype. The increase in seizure thresholds as a result of the MS paradigm was not surprising, given that the MS pups received increased levels of maternal care. Increased levels of maternal behaviors, particularly licking, have been correlated with increased long-term resistance to stress (Ladd et al. 2000). Increased maternal care has been shown to decrease reactivity in the hippocampus and cortex, providing a possible mechanistic explanation for an increased seizure resistance (Leussis and Heinrichs 2009). But why the stronger effect in *Scn8a*$^{med/+}$ mutants? It is known that *Scn8a* is one of the most ubiquitously expressed VGSCs (Krzemien et al. 2000), and expression levels in mice begin increasing around P10 (Gazina et al. 2010). The timing of *Scn8a* expression coincides with the period of early life manipulation in this study. Given the fact that early life experience has been shown to alter excitability in the hippocampus, a key region for seizure propagation, it is plausible to consider that in the *Scn8a*$^{med/+}$ mutants, excitability is being modified by two separate forces: the early life experience and the early life expression of a mutant channel. Our results suggest that in the case of early life enrichment, as defined by increased maternal care, the resulting effects on excitability are synergistic. In other words, the adult MS *Scn8a*$^{med/+}$ mutants are doubly protected from induced seizure generation, first by the mutation itself which has been proposed to decrease overall network excitation (Martin et al. 2007), and secondly by a possible

reduction in hippocampal excitability caused by the increased maternal care (Leussis and Heinrichs 2009). Future studies should investigate changes in excitability in neural structures key to the propagation of generalized seizure activity, such as the hippocampus and amygdala, in *Scn8a*$^{med/+}$ mutants who have undergone altered early life experiences.

We also observed genotype findings in this study which contradict our previous results, such as the increased CORT response to a stressor and the increased amount of floating in the forced swim test seen in the male *Scn8a*$^{med/+}$ mutants in this study. A plausible explanation for this contradiction lies in the study design. All animals, both in the MS group and in the DH group, underwent daily handling from P2 until P28 for daily weight measurements, and then weekly handling until P56 for weekly weight measurements. Daily handling of rodents is known to affect behavioral outcomes (Schmitt and Hiemke 1998). This may explain why these specific genotype differences were observed in both the MS and DH groups in this study, but not in a previous group of *Scn8a*$^{med/+}$ mutants that were not exposed to daily handling (Sawyer et al. 2014). Nevertheless, it appears that daily handling is sufficient to unmask genotype differences previously unobserved, and this finding underscores the importance of early life experience on the development of phenotypic traits in adulthood. Results from this study would suggest that in a different rearing environment, male *Scn8a*$^{med/+}$ mutants might have a more reactive HPA axis and exhibit depressive-like behaviors.

3.5.6 Sex-Genotype Interactions

Another interesting aspect of this study was the results from the novel cage paradigm, a test designed to measure the response to novelty. Not only did the males and females perform differently, but the differences depended on the genotype. WT females displayed less activity and exploration than the males, while *Scn8a*$^{med/+}$ females were more active and exploratory. Interestingly, the WT females performed as expected, since it has been shown that male mice have a greater preference for novelty than females (Frick and Gresack 2003). This raises the question of how the *Scn8a* mutation is interacting with sex to create a phenotype of increased preference for novelty in the female *Scn8a*$^{med/+}$ mutants. It has been shown that female sex hormones are neuroactive and can influence excitability within the brain (Herzog 2007). It is possible that alterations in excitability within certain networks responsible for the response to a novel environment have undergone modification in the *Scn8a*$^{med/+}$ females by two forces, the *Scn8a* mutation and the actions of female sex hormones, leading to an altered behavioral response in the *Scn8a*$^{med/+}$ females, but not in the WT females. This two-hit alteration of excitability within the female brain may also explain why only the *Scn8a*$^{med/+}$ females showed increased latencies to picrotoxin-induced seizures and flurothyl-induced seizures when compared to both WT females as well as *Scn8a*$^{med/+}$ males. As the hippocampus has been implicated in mediating both the response to novelty and the propagation of generalized seizures, future studies could look at the level of excitability in the hippocampus in *Scn8a*$^{med/+}$ females as compared to WT females and *Scn8a*$^{med/+}$ males. Furthermore, it would be interesting to see if mutations in a VGSC can affect levels of

gonadal hormones, something that was not tested in the current study, but that could also provide an explanation for these results.

3.6 Conclusion

Overall, these findings suggest that early life experience can have divergent effects dependent on both genetics and sex. These findings point toward a very complex interplay between genetics, sex, and the environment. Future studies could attempt to narrow and define in more detail specific findings of the current study. We have demonstrated that not only does a VGSC gene result in a novel phenotype characterized by alterations in seizure activity and behavior, but it appears that mutations in *Scn8a* can also interact with other developmental processes, such as the shaping of the brain by gonadal hormones, to influence adult behavior. Furthermore, the adult phenotype that occurs as a result of a single VGSC mutation depends on environmental factors and rearing conditions. Results from this study may provide insight into the complex phenotypic variation seen in genetic epilepsies, both in humans and in rodent models (Ottman 2005; Frankel 2009). While reductionist approaches such as the study of a single VGSC mutation offer a wealth of information, it should be kept in mind that phenotypic variation arises from complex, multifaceted layers of other interacting factors such as sex and early life experience.

3.7 Acknowledgements

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CHAPTER 4: DYSFUNCTION OF THE VOLTAGE-GATED SODIUM CHANNEL GENE *SCN1A* ALTERS BEHAVIOR, BUT NOT THE SEIZURE RESPONSE TO STRESS

4.1 Abstract

Mutations in the voltage-gated sodium channel (VGSC) gene *SCN1A* are responsible for a number of epilepsy disorders, including genetic epilepsy with febrile seizures plus (GEFS+) and Dravet syndrome (DS). In addition, dysfunction in *SCN1A* is increasingly being linked to hyperactivity, social deficits, and cognitive disabilities. We have previously reported that mice heterozygous for the *SCN1A* R1648H mutation identified in a GEFS+ family have infrequent spontaneous seizures, susceptibility to chemically- and hyperthermia-induced generalized seizures, and abnormalities in sleep. In the present study, we characterized the behavior of heterozygous knock-in mice expressing the *SCN1A* R1648H mutation (*Scn1a*$^{RH/+}$) and the effect of stress on spontaneous and induced seizures. We also examined the effect of an *Scn1a* mutation on hypothalamic-pituitary-adrenal (HPA) axis response. We have confirmed our previous findings that *Scn1a*$^{RH/+}$ mutants are hyperactive, and also identified deficits in social behavior, spatial memory, cued fear conditioning, prepulse inhibition (PPI), and risk assessment. Furthermore, while exposure to a stressor did reduce seizure thresholds, the effect seen in the *Scn1a*$^{RH/+}$ mutants is similar to that seen in wildtype (WT) littermates. *Scn1a* dysfunction also does not appear to alter HPA function in adult animals. Our results suggest that the behavioral abnormalities associated with *Scn1a* dysfunction encompass a wider range of phenotypes than previously reported. Also, factors such as stress exposure may alter disease severity in patients with *SCN1A* mutations.

4.2 Introduction

Mutations in the voltage-gated sodium channel (VGSC) gene *SCN1A* are responsible for several epilepsy subtypes, including Dravet syndrome (DS) (Claes et al. 2001), genetic epilepsy with febrile seizures plus (GEFS+) (Escayg et al. 2000), and intractable childhood epilepsy with generalized tonic-clonic seizures (GTCSs) (Mulley et al. 2005; Fujiwara 2006). The prevailing hypothesis that these mutations lead to seizure generation by specifically reducing the excitability of inhibitory interneurons, thereby decreasing overall network inhibition (Yu et al. 2006; Ragsdale 2008; Tang et al. 2009; Martin et al. 2010), has recently been confirmed by a study directly linking the activity of *Scn1a* in parvalbumin interneurons to seizure susceptibility (Ogiwara *et al.* 2007; Cheah *et al.* 2012; Dutton *et al.* 2012). DS is characterized by febrile seizures in the first year of life, with progression to partial and/or generalized afebrile epilepsy, moderate to severe intellectual disability, and ataxia (Mulley *et al.* 2005; Fujiwara 2006; Wolff *et al.* 2006). The less severe GEFS+ phenotype includes febrile seizures that persist beyond six years of age and the development of afebrile generalized or partial seizures during adulthood (Scheffer and Berkovic 1997). Within a GEFS+ family, affected members with the same mutation exhibit a wide range of epilepsy subtypes and severities, suggesting that other genetic, developmental, and/or environmental factors may play a role in the development and expression of GEFS+ (Scheffer and Berkovic 1997). There is anecdotal evidence to suggest that stress can precipitate seizures in some patients with GEFS+ (Grant and Vazquez 2005; Baulac et al. 2008), and the avoidance of stressful situations is recommended for Dravet syndrome patients as well (Ceulemans et al. 2004).

In addition to epilepsy, some individuals with *SCN1A* mutations display neuropsychiatric abnormalities, such as anxiety disorders, affective disorders and autism spectrum disorders (ASDs) (Weiss *et al.* 2003; Osaka *et al.* 2007; Mahoney *et al.* 2009; O'Roak *et al.* 2012), suggesting a wider range of phenotypes associated with *SCN1A* mutations. ASDs have recently been more broadly linked with deficiencies in inhibitory signaling (Chao et al. 2010; Paluszkiewicz et al. 2011), possibly providing a mechanistic basis for the relationship with altered *SCN1A* function. Recently, autistic-like behavior has been described in a *Scn1a* knockout mouse and a mouse with a nonsense mutation in *Scn1a*, both mouse models of DS (Han et al. 2012; Ito et al. 2012), providing further support for a link between *SCN1A* dysfunction and autistic-like behaviors.

The *SCN1A* mutation, R1648H, was identified in a large family with GEFS+ (Escayg et al. 2000). Our lab has generated a mouse model of GEFS+ by knocking in the R1648H mutation into the orthologous mouse *Scn1a* gene (Martin et al. 2010). Heterozygous mutants ($Scn1a^{RH/+}$) exhibit infrequent spontaneous generalized seizures and lower thresholds to flurothyl- and hyperthermia-induced seizures, whereas homozygous mutants ($Scn1a^{RH/RH}$) exhibit frequent spontaneous seizures and premature lethality. In addition to the seizure phenotype, we have also found sleep abnormalities in the $Scn1a^{RH/+}$ mutants (Papale et al. 2013), further expanding the range of phenotypes associated with *SCN1A* mutations. Previous work on the $Scn1a^{RH/+}$ mutants was performed in mice on a mixed 129X1/SvJ x C57BL/6J background (N2 generation). In order to lessen the influence of a mixed genetic background on observed phenotypes, the $Scn1a^{RH/+}$ line was backcrossed on C57BL/6J for twelve generations (N12).

The goal of this study was threefold. First, we re-examined the influence of the *Scn1a* R1648H mutation on seizure threshold and life-span in mice at the N12 generation. Second, we examined the behavioral characteristics of *Scn1a*$^{RH/+}$ mutants. Finally, we examined the effect of stress on seizure occurrence and asked if the *Scn1a*$^{RH/+}$ mutation was sufficient to alter the functioning of the hypothalamic-pituitary-adrenal (HPA) axis, a key player in the physiological stress response.

4.3 Methods

Animals

Scn1a^{RH} mice were generated as previously described (Martin et al. 2010) and were initially on a mixed 129X1/SvJ x C57BL/6J background. *Scn1a*$^{RH/+}$ males were backcrossed to C57BL/6J females to advance the generation on the C57BL/6J background until the 12th backcross generation, designated the N12 generation, was reached. Survival and pre-weaning weights were monitored. Male mice, 3-4 months old, were used for all other experiments. Wildtype (WT) littermates were used as controls for all experiments to minimize variation due to differences in genetic background and rearing conditions. Mice were group housed after weaning (P21) in ventilated cages under uniform conditions with a 12/12 hour light/dark cycle with lights on at 7 am and lights off at 7 pm. Mice remained group housed until one week prior to testing, at which time they were single-housed. Food and water were available *ad libitum*. All testing was done between 8 am and 1 pm to minimize variation due to circadian factors. All

experiments were approved by the Emory University Institutional Animal Care and Use Committee (IACUC).

Genotyping

Genotyping of *Scn1a*^{RH} mutants was performed on tail DNA using primer pair FRH (5'-TTGATGACTTCTTCACTGATTGAT) and RRH (5'-AGAGGCTCTGCACTTTCTTC). PCR amplification was performed by a 2 min denaturation at 94°C followed by 32 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. The 591-bp PCR product was digested with EcoRI to distinguish between the wildtype (WT; 591 bp) and mutant (461 bp) alleles. Products were separated by gel electrophoresis on a 1.2% agarose gel. Heterozygous *Scn1a*$^{RH/+}$ mutants were identified by the presence of both a WT band and a mutant band. Homozygous *Scn1a*$^{RH/RH}$ mutants were identified by the absence of the WT band.

Life-Span and Weights

In order to characterize the effect of the *Scn1a* R1648H mutation on the more congenic C57BL/6J background, 20 wildtype C57BL/6J females were bred with two different heterozygous *Scn1a*$^{RH/+}$ males (N11 generation) to obtain twenty litters of N12 offspring (n=146 offspring). The litters were of normal size (an average of 7.3 pups per litter) with expected Mendelian ratios for the offspring. Beginning on postnatal day 2 (P2), all pups were toe clipped and weighed daily until weaning (P21). After weaning, animals were weighed weekly until postnatal week 8. One final weight measurement was taken at postnatal day 90 prior to the initiation of any other experiments. All mice were monitored daily from P2 until P90.

Stressor

The stressor used for these experiments is the same as the one described in Chapter 2 (Section 2.3).

EEG Analysis of Spontaneous Seizure Activity

EEG and EMG electrodes were placed as described in Chapter 2 (Section 2.3). Following 24 hours of habituation, continuous EEG and EMG data along with simultaneous digital video recording were collected for a 7-day baseline period in 8 male *Scn1a*$^{RH/+}$ mutants. Following the 7-day period of baseline recording, the seizure response to acute restraint stress was determined by placing animals into modified restraint tubes (50-mL conical plastic tube with a 1.5x1.5 cm^2 hole cut out for EEG headcap placement) for 20 minutes. Continuous EEG data were collected for the 20-minute restraint period as well as for the 6-day period following the acute restraint. Seizure response to chronic restraint stress was then determined by placing animals into modified restraint tubes for 20 minutes each day over a 6-day period. EEG data was collected for an additional four days after the completion of stress exposure on the sixth day. All data were analyzed for the number of seizures as characterized by polyspike activity accompanied by stereotypic seizure behaviors and a refractory period.

Flurothyl Seizure Induction

Flurothyl seizure induction was performed as described in Chapter 3 (Section 3.3).

Picrotoxin Seizure Induction

Picrotoxin seizure induction was performed as described in Chapter 2 (Section 2.3).

Open Field Test

The open field test was performed as described in Chapter 3 (Section 3.3).

Object Recognition Test

Testing was conducted in the open field square arena (60x60 cm^2). In total, four colored plastic objects were used for testing: a bear, a dog, a duck, and a grenade. The objects were similar in size (~10x6x6 cm) but differed in color and texture. The test session consisted of four consecutive trials, each 10 minutes in duration. Twelve mice of each genotype were tested. After completing each trial, the animal was returned to the home cage for five minutes. In trial 1, the mouse was placed in the empty open field and allowed to freely explore the testing arena. For trial 2, three objects were introduced into the open field in three designated locations at least 5 cm from the walls. The selection of the objects and their placement within the open field was randomized for each mouse. Each mouse was placed in the center of the box and allowed to freely explore the testing arena. For trial 3, one object from trial 2 was replaced with a novel object in the same location (novel object). In trial 4, a different object from trial 2 was relocated to a novel fourth location (relocated object). Throughout trials 2, 3, and 4 there was one object that was never changed nor moved (control object). Mice were videotaped and behavior was scored using the ANY-maze Video Tracking System (Stoelting Co). The time spent exploring each object within the first five minutes of the trial was scored. A mouse was considered to be exploring an object when it was facing the object at a close distance (≤5 mm) or when the mouse's nose or front paws were in contact with the object. The discrimination ratio was calculated by taking the time spent exploring either the novel or

relocated object divided by the total time spent exploring both the manipulated object and the control object, multiplied by 100.

Contextual and Cued Fear Conditioning

On the first day, mice (12 *Scn1a*$^{RH/+}$ and 12 WT littermates) were placed into a fear conditioning apparatus (17.5 cm x 17.5 cm x 30 cm (h), Colbourn, Pennsylvania) and allowed to explore the enclosure for 3 minutes. Following this habituation period, three conditioned stimulus (CS)-unconditioned stimulus (US) pairings were presented with a 1-minute intertrial interval. The CS consisted of a 20-second 85 dB tone and the US consisted of 2 seconds of a 0.5 mA footshock, which co-terminated with each CS presentation. Shock was delivered via a Precision Animal Shocker (Colbourn) connected to the fear-conditioning chamber. One minute following the last CS-US presentation, animals were returned to their home cage. On day 2, mice were tested for contextual fear conditioning by placing them into the same chamber used for conditioning on day 1. No shocks were administered during the context test and mice were allowed to remain in the apparatus for eight minutes. On day 3, mice were tested for cued fear conditioning by placing the animals into a novel compartment and administering the CS. Mice were first allowed to freely explore the novel context for 2 minutes. Following this habituation period, the 85 dB tone was presented for 6 minutes. No shocks were administered during the tone test. During all three days, animals were recorded via a camera and the amount of freezing was measured using software provided by Colbourn. These experiments were performed and the data collected by Dr. Jason Schroeder in Dr. David Weinshenker's laboratory (Emory University Rodent Behavioral Core). We performed the data analysis.

Baseline Startle and Prepulse Inhibition (PPI)

Startle was measured using a San Diego Instruments SR-Lab Startle Response System (San Diego, CA). Mice (12 *Scn1a*$^{RH/+}$ and 12 WT littermates) were placed into the Plexiglas holders and allowed to acclimate to the chamber and background white noise (60 dB) for five minutes. After the acclimation period, randomized trials of either startle alone (117 dB for 40 ms), no stimulus, or startle preceded by one of four prepulse intensities (70, 72, 76, or 80 dB) were administered. A total of 48 trials were run (8 trials of each of the different conditions) with a random intertrial interval. The maximum startle amplitude was measured during the first 100 ms following the pulse presentation for each trial. The average startle amplitude to the startle alone stimulus was used to calculate the prepulse inhibition.

Predator Odor Exposure

The predator odor experiment was conducted as described in Chapter 2 (Section 2.3) using a 1:100 dilution of the TMT.

Olfactory Discrimination Tests

The ability to detect three types of odors (food, social, and aversive) was tested using a three-chambered apparatus. Each of the three chambers measured 20 cm x 40 cm x 22 cm (h). The partitions separating the chambers had a square opening (5 cm x 5 cm) in the bottom center. A covered petri dish with holes to release the odor was used to hold the odors and the control odor (water). For the food test, a mix of seed and nuts (Kaytee) was placed in the test dish. For the social test, soiled bedding from an unfamiliar male mice was placed in the test dish. For the aversive odor, 10 µL of diluted TMT (1:100 dilution

in ethanol) was placed on a piece of filter paper inside the test dish. The test dish containing the food, bedding, or TMT was placed in the corner in one of the outer chambers. The control dish containing water was placed in the corner in the opposite outer chamber. The sides containing the test odor and the control odor were alternated between animals. Animals (12 *Scn1a*$^{RH/+}$ mutants and 12 WT littermates) were placed into the middle chamber and allowed to freely explore for ten minutes. Animals were videotaped, and the video was later scored by a user blind to the genotypes using the ANY-Maze Video Tracking System (Stoelting Co). For the food and social odors, the time spent in a 6-cm zone around the two dishes was scored. For the aversive odor, a transparent plexiglas lid was added to the chamber to prevent diffusion of the highly volatile TMT throughout the chamber. Analysis of the aversive odor was done by calculating the percent of time spent in the chamber containing the aversive odor as compared to the time spent in the other two chambers. A score of 33.3% would represent chance occurrence.

Visual Cliff Avoidance Task

The visual cliff avoidance task was conducted in a rectangular clear Plexiglas box with a clear bottom. White paper was taped around the sides to make them opaque and to prevent the mouse from seeing the behavioral test administrator in the room. The box was secured so that half of the floor of the box overhung the table top to create an appearance of a ledge. A checkerboard tablecloth draped from the table top to the floor served to enhance the visual appearance of the cliff. An opaque neutral zone (10 cm x 10 cm) was located in the center of the box floor. The side overhanging the table top was designated the “cliff” zone and the side sitting on the table was designated the “safe” zone. The

neutral zone lay between the cliff zone and safe zone. Mice (12 *Scn1a*$^{RH/+}$ mutants and 12 WT littermates) were placed onto the neutral central region and allowed to freely explore for 10 minutes. Mice were recorded and the video was analyzed using the ANY-Maze Video Tracking System (Stoelting Co). Time spent in each zone was measured. After observing that some mice that ventured onto the cliff side were staying close to the opaque walls of the apparatus while others would walk into the open area above the drop-off, time spent in the center zone of the cliff side was also measured. The center zone of the cliff side was designated as the area of the cliff zone that was at least 6 cm away from any wall.

Vision Testing

Visual function was tested using the virtual optokinetic system (OptoMotry system; Cerebral Mechanics, Lethbridge, AB, Canada) as previously described (Douglas et al. 2005). Briefly, the mice (5 *Scn1a*$^{RH/+}$ mutants and 5 WT littermates) were placed on a platform at the center of a virtual-reality chamber composed of four computer monitors that display vertical sine wave grating rotating at a speed of 12°/s. The experimenter monitored the mice real-time through a video camera situated above the animal and noted the presence or absence of reflexive head movements (tracking) to the gratings. The experimenter also manually tracked the head of the mouse to align the center of the virtual cylinder to the viewing position of the mouse. For visual acuity assessment, the grating started at a spatial frequency of 0.042 cyc/deg with 100% contrast and increased with a staircase procedure. The maximum spatial frequency capable of eliciting head tracking was determined using the OptoMotry system. This experiment was performed

and the data collected by Megan Prunty in Dr. Machelle Pardue's laboratory. We performed the data analysis.

Social Interaction

Social interaction was tested using a three-chambered apparatus. Each of the three chambers measured 20 cm x 40 cm x 22 cm (h). The partitions separating the chambers had a square opening (5 cm x 5 cm) in the bottom center. A cylindrical wire cage (10 cm diameter, Pencil Holder, Design Ideas) was used as an inanimate object or the cage housing a stranger mouse. In the first 10-minute session, a test mouse (12 *Scn1a*$^{RH/+}$ mutants and 12 WT littermates) was placed in the center chamber. Two empty wire cages were placed in the left and right chambers. The mouse was allowed to freely explore the apparatus. In the second 10-minute session, an age- and sex-matched C57BL/6J mouse (Stranger) that had never been exposed to the test mouse, was place in one of the two wire cages. The wire cage on the other side remained empty. The test mouse was again placed in the center chamber and allowed to freely explore. For the third 10-minute session, a second age- and sex-matched C57BL/6J stranger mouse (Novel Mouse) was placed in the wire cage which previously served as an empty cage. Thus, the test mouse would now have a choice between a familiar mouse and a novel mouse. The test mouse was again placed in the center chamber and allowed to freely explore for 10 minutes. All three stages were videotaped, and the video was analyzed by an experimenter blind to the animals' genotype using the ANY-Maze Video Tracking System (Stoelting Co). Time spent in each chamber, and time spent within a 5-cm radius proximal to each wire cage was measured.

Mating Calls

The home cages of male subjects (12 *Scn1a*$^{RH/+}$ mutants and 12 WT littermates) were placed in a single-walled, 127 cm x 83.8 cm x 83.8 cm sound-attenuating box (ETS-Lindgren, Cedar Park, TX, USA) with the lids removed. A Bruel & Kjær (Nærum, Denmark) microphone with a ¼" membrane diameter was positioned over the cage, ~15 cm above the cage floor, with the membrane pointed downward into the cage. Under red light, animals were monitored from outside the sound box via a video camera and bat detector (Ultra Sound Advice, London, UK). After a ~1 minute period of baseline recording, an unfamiliar adult female mouse was introduced to the cage and animals were allowed to freely interact. Using the Bruel & Kjær mic, in conjunction with Tucker-Davis Technologies data acquisition hardware (Alachua, FL, USA) and custom MATLAB programs, vocalizations were recorded at 223 kilosample/second. After 10 minutes, the cage was removed from the sound box and the mice were separated. Vocalizations were extracted from the sound recordings offline, using custom MATLAB programs. First, the file was high-pass filtered about 25 kHz to eliminate any low-frequency sounds caused by the animals' movements inside the cage. The file was then denoised by subtracting a background noise level (calculated from a ~0.5 second portion of the sound file during which no vocalizations occurred) from the entire sound file. Next, putative calls were identified by identifying regions where the power exceeded a manually-set threshold (set per animal by an experimenter blind to the animal's genotype) for at least 5 ms. If consecutive calls were separated by fewer than 30 ms, they were grouped as one. Though extraction was performed in an automated fashion, an experimenter validated call boundaries by visualizing spectrograms of a portion of the extracted calls. This

experiment was performed and the data collected by Katy Shepard in Dr. Robert Liu's laboratory. We performed the data analysis.

Corticosterone Measurements

Blood was collected at 7 am and 7 pm in order to coincide with the expected nadir and peak of circulating corticosterone (CORT) levels. All animals were sampled once at 7 am and once at 7 pm, with each sampling separated by two days. A week after the last diurnal blood draw, the effect of an acute stressor on the HPA axis and on subsequent evening CORT levels was measured. Animals were subjected to a 20-min acute restraint stress in the morning between the hours of 8-9 am. Blood was drawn immediately after the restraint stress and then again at 7 pm for a post-stress evening CORT measurement. For all basal CORT measurements, blood was obtained within 1 minute of cage disturbance. Blood was collected from the facial vein into Microvette CB 300 Z tubes (Starstedt) and then allowed to clot for 1 hour at room temperature. The serum was then separated by centrifugation at 5600 rpm for 15 minutes at 4°C and stored at -80°C until analysis. Serum CORT levels for the diurnal blood draws were assayed using a commercial radioimmunoassay (RIA) kit (MP Biomedicals) according to the manufacturer's instructions.

Statistics

The data are reported as mean ± standard error of the mean (SEM). When comparing only two groups of unrelated parametric data, the data were analyzed using the Student t-test. Parametric data sets with two or more groups/factors to be compared were analyzed using two-way analysis of variance (ANOVA) if there was only one measurement per

animal or two-way repeated measures ANOVA (rANOVA) if there were multiple data values from a single animal. Following the ANOVA analyses, the Tukey pairwise comparison test was used to further distinguish among groups. Nonparametric data were analyzed using the Mann-Whitney Rank Sum test. Dichotomous data were analyzed using the Fisher Exact test. All results were considered statistically significant if $p<0.05$.

4.4 Results

4.4.1 N12 *Scn1a*^{RH} mutants exhibit a shortened life span, spontaneous seizures, and a reduced latency to flurothyl-induced seizures

We began by characterizing the *Scn1a* R1648H mutation on an N12 congenic C57BL/6J background. Beginning at P10, *Scn1a*$^{RH/RH}$ mutants began to show slower weight gain, with weight loss and decreased survivalability beginning around P16 (**Fig. 4.1**A,B). Homozygous *Scn1a*$^{RH/RH}$ mutants have a statistically significant shorter lifespan when compared to both *Scn1a*$^{RH/+}$ mutants (log rank statistic 111.555, $p<0.001$) and WT littermates (log rank statistic 76.407, $p<0.001$) (Fig. 1A) as we reported previously with the N2 generation (Martin et al. 2010). However, the N12 heterozygous *Scn1a*$^{RH/+}$ mutants also have a significantly different life span than WT littermates (log rank statistic 5.698, $p<0.05$, **Fig 4.1**A), a novel finding of this study. While there is a significant main effect of genotype when comparing pre-weaning weights ($F_{(2,129)}=29.086$, $p<0.001$), there was also a significant interaction between genotype and age ($F_{(38,2402)}=97.646$, $p<0.001$), with only the *Scn1a*$^{RH/RH}$ mutants showing a slower growth rate beginning around P10 and a subsequent loss of weight around P16 (**Fig 4.1**B).

After weaning, there were too few surviving *Scn1a*$^{RH/RH}$ mutants to include in analysis of post-weaning weights. There were no statistically significant differences in the post-weaning weights of male *Scn1a*$^{RH/+}$ mutants as compared to male WT littermates (**Fig. 4.1**C). Due to sex differences in weights after weaning, females were analyzed separately post-weaning. Like the males, there were no differences in post-weaning weights between heterozygous female mutants and WT littermates (data not shown).

Spontaneous seizures were observed in both heterozygous *Scn1a*$^{RH/+}$ mutants and homozygous *Scn1a*$^{RH/RH}$ mutants during daily weighing sessions as well as during routine mouse colony management procedures. Eight *Scn1a*$^{RH/+}$ were analyzed with EEG recordings to characterize spontaneous seizure activity. Within the week of continuous baseline recording, only 2 of the 8 animals had spontaneous seizure activity, reinforcing the previous observation that heterozygous *Scn1a*$^{RH/+}$ mutants have infrequent spontaneous seizures (Martin et al. 2010).

We also asked whether the N12 *Scn1a*$^{RH/+}$ mutants would exhibit increased susceptibility to flurothyl-induced seizures as was previously observed in the N2 *Scn1a*$^{RH/+}$ mutants. Like the N2 mutants, there was no difference in latency to the myoclonic jerk, but *Scn1a*$^{RH/+}$ mutants showed a significantly lower threshold to the flurothyl-induced GTCS ($t_{(27)}$=2.844, $p<0.01$, **Fig. 4.1**D).

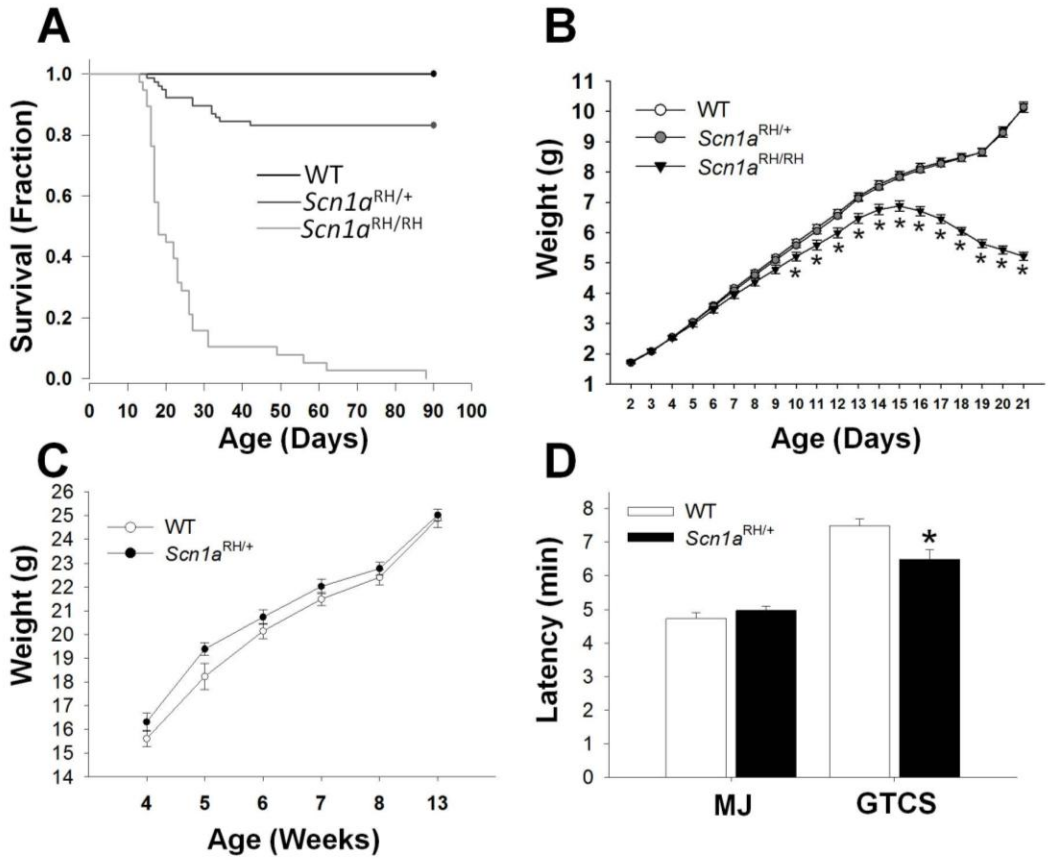

Figure 4.1 Characterization of the N12 congenic C57BL/6J *Scn1a* ^{R1448H} mutant line. (A) Both *Scn1a*$^{RH/+}$ mutants (n=77) and *Scn1a*$^{RH/RH}$ mutants (n=38) exhibited a statistically significant reduction in life span compared to WT littermates (n=31); however, the reduction in the lifespan of the *Scn1a*$^{RH/+}$ mutants was much less severe than the reduction seen in *Scn1a*$^{RH/RH}$ mutants. **(B)** The pre-weaning weight gain of *Scn1a*$^{RH/+}$ mutants (n=76) and WT littermates (n=31) are indistinguishable; however, *Scn1a*$^{RH/RH}$ mutants (n=37) show a significant reduction in growth rate around P10, and a reduction in body weight beginning around P16. *p<0.05 vs. WT and *Scn1a*$^{RH/+}$. **(C)** Post-weaning weights of male *Scn1a*$^{RH/+}$ mutants (n=34) are not significantly different from the post-weaning weights of male WT littermates (n=17). **(D)** There is no difference in latency to a myoclonic jerk (MJ) between *Scn1a*$^{RH/+}$ mutants (n=14) and WT littermates (n=15); however, *Scn1a*$^{RH/+}$ mutants show a signification reduction in latency to a generalized tonic-clonic seizure (GTCS). *p<0.05 vs. WT. Error bars represent SEM.

4.4.2 *Scn1a*$^{RH/+}$ mutants are hyperactive

We previously demonstrated that *Scn1a*$^{RH/+}$ mutants have increased locomotor activity in a novel environment (Purcell et al. 2013). We have extended this finding to show that the *Scn1a*$^{RH/+}$ mutant mice show hyperactivity in a number of behavioral tasks. In the open field test, *Scn1a*$^{RH/+}$ mutants travel a greater total distance ($t_{(21)}$=-2.649, $p<0.05$, **Fig. 4.2**A), travel at a higher average speed ($t_{(21)}$=-2.665, $p<0.05$, **Fig. 4.2**B), and spend less time immobile during the task ($t_{(21)}$=3.867, $p<0.001$, **Fig. 4.2**C) as compared to WT littermates. There were no significant differences in the amount of time the *Scn1a*$^{RH/+}$ mutants spent in the center zone as compared to WT littermates (**Fig. 4.2**D), nor was there a difference in latency to enter the center zone (data not shown), suggesting that the mutants exhibit normal anxiety levels. The hyperactive phenotype of the *Scn1a*$^{RH/+}$ mutants has now been replicated in a number of other behavioral tasks (**Appendix B1**).

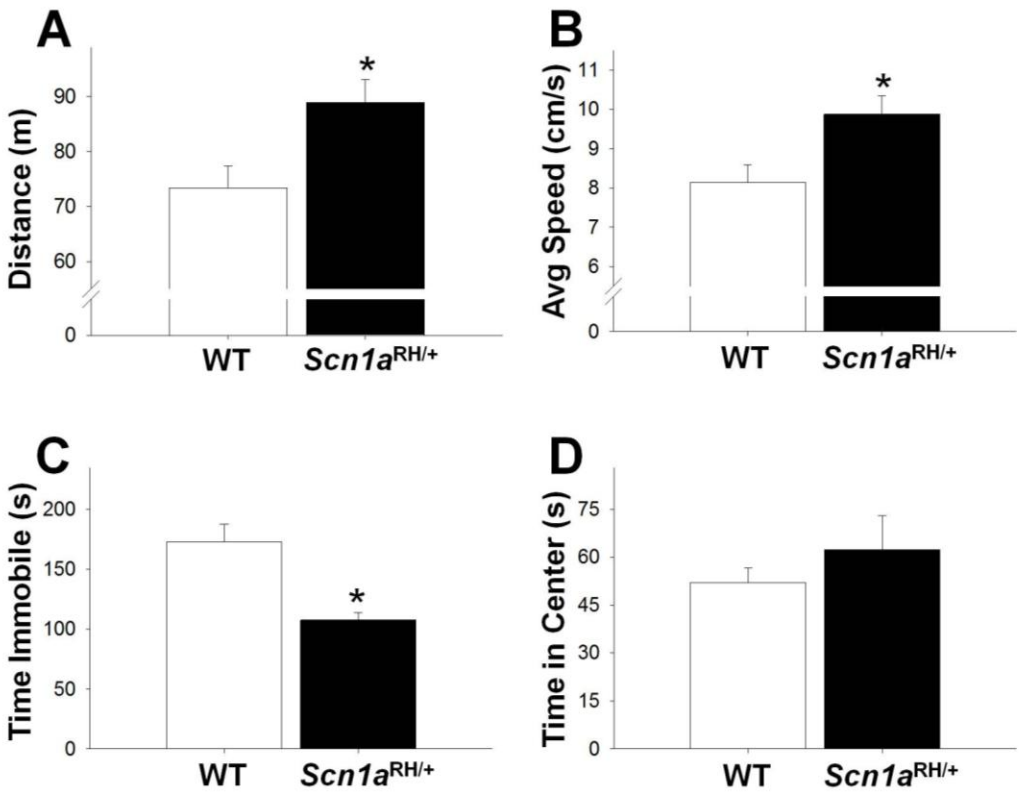

Figure 4.2 *Scn1a*$^{RH/+}$ mutants are hyperactive. *Scn1a*$^{RH/+}$ mutants (A) travel a farther distance, (B) travel at a higher average speed, and (C) spend less time immobile while in the open field as compared to WT littermates. (D) There are no differences between genotypes in the time spent in the center zone of the open field. n=12 all groups; *p<0.05 vs. WT; Error bars represent SEM.

4.4.3 *Scn1a*$^{RH/+}$ mutants show mild cognitive impairment and deficits in sensorimotor gating

An object recognition test was used to assess learning and memory in the *Scn1a*$^{RH/+}$ mutants. To evaluate object recognition memory, the discrimination ratio was calculated by taking the time in trial 3 spent exploring the novel object and dividing it by the total time spent exploring the novel and the control object (*i.e.* novel/(novel + control) x 100). This score provides a measurement of the subject's memory for an object's identity, and since mice have an innate preference for novelty (Antunes and Biala 2012), it is expected that they will spend more time with the novel object (a ratio significantly greater than 50%). Indeed, both *Scn1a*$^{RH/+}$ mutants and WT littermates spent more time with the novel object compared with the control object (one-tailed *t* test, WT: $t_{(11)}$=8.452, p<0.001, *Scn1a*$^{RH/+}$: $t_{(11)}$=2.839, p<0.05; **Fig. 4.3**A). While the WT littermates spent a greater percentage of time with the novel object than the *Scn1a*$^{RH/+}$ mutants (WT: 74.4 ± 2.9%, *Scn1a*$^{RH/+}$: 65.2 ± 5.3%), the difference between genotypes did not reach statistical significance ($t_{(22)}$=1.519, p=0.143).

To evaluate spatial object memory, the discrimination ratio was calculated by taking the time in trial 4 spent exploring the relocated object and dividing it by the total time spent exploring the relocated and the control object (*i.e.* relocated/(relocated + control) x 100). As before, a score significantly greater than 50% would represent a memory of the object's original spatial position. Mice have been shown to be able to form memories of not only the objects themselves, but also their location in space (Dere et al. 2005). Our results show that the WT animals, but the not the *Scn1a*$^{RH/+}$ mutants, are able to remember the spatial location of the objects (one-tailed *t* test, WT: $t_{(11)}$=2.349,

p<0.05, *Scn1a*$^{RH/+}$: $t_{(10)}$=0.917, p=0.381; **Fig. 4.3**A). Thus, while both groups appear to remember the objects' identities equally well, the mutants show a deficit in spatial learning and memory in this task.

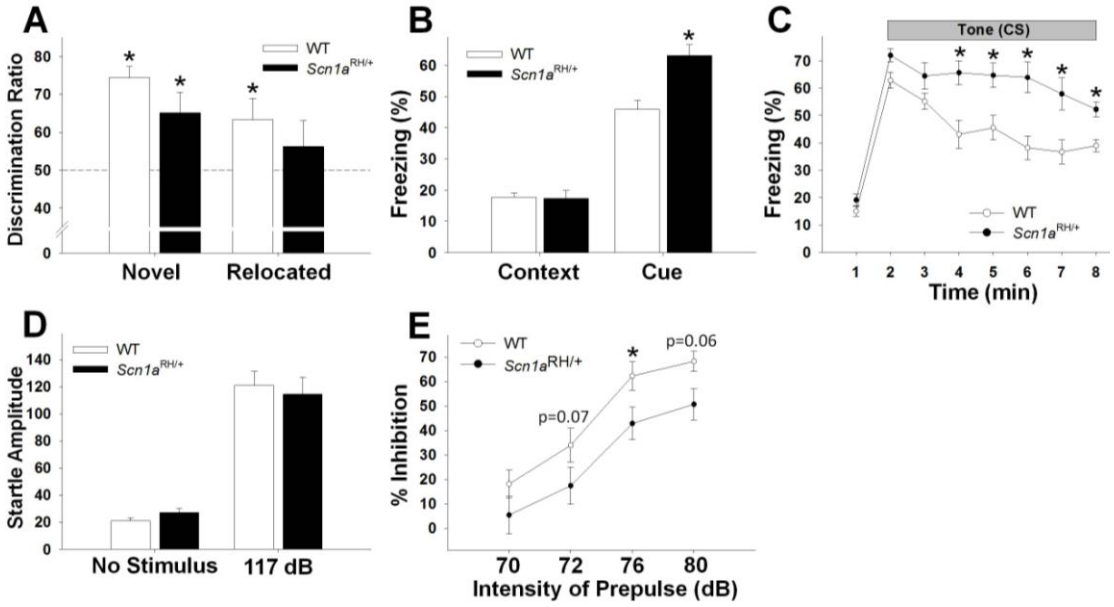

Figure 4.3 ***Scn1a*$^{RH/+}$ mutants show mild cognitive deficits and impairments in sensorimotor gating. (A)** Both *Scn1a*$^{RH/+}$ mutants and WT littermates show a preference for a novel object, but only the WT littermates show a preference for a relocated object. The value shown is the discrimination ratio, calculated as the time spent exploring the novel or relocated object divided by the total time exploring both the manipulated object and the control object, multiplied by 100. The dotted line represents a performance at chance level (50%). *p<0.05, one-tailed t test vs. 50% chance. **(B)** A contextual and cued fear conditioning task results in no differences in contextual freezing between *Scn1a*$^{RH/+}$ mutants and WT littermates, but *Scn1a*$^{RH/+}$ mutants show significantly more cued freezing. *p<0.05 vs. WT. **(C)** A closer examination of freezing during the cued fear conditioning paradigm shows that during the six minutes that the tone (conditioned stimulus, CS) was played but no shock was administered, the WT littermates showed a reduction in their freezing behavior, while *Scn1a*$^{RH/+}$ mutants continued to maintain high levels of freezing. *p<0.05 vs. WT. **(D)** There are no differences in the amplitude of the startle response between *Scn1a*$^{RH/+}$ mutants and WT littermates. **(E)** *Scn1a*$^{RH/+}$ mutants show decreased pre-pulse inhibition in the startle response compared to WT littermates, particularly at the higher decibel levels of the prepulse tone. *p<0.05 vs. WT. n=12 all groups. Error bars represent SEM.

To measure their ability to learn and retain fear memories, we tested the *Scn1a*$^{RH/+}$ mutants with a fear conditioning paradigm. On the first day, mice were training to associate a tone (CS) with a mild foot shock (US) using a delayed fear conditioning protocol in which the US was administered at the end of the CS presentation. There were no differences in the time spent freezing on the day of training between mutants and WT littermates (data not shown). On day 2, mice were placed back into the same context and freezing was measured over a period of six minutes. There were no differences in the amount of freezing during the contextual fear conditioning task between *Scn1a*$^{RH/+}$ mutants and WT littermates ($t_{(22)}$=0.113, p=0.911; **Fig. 4.3**B, Context).On day 3, mice were placed in a novel environment and the tone (CS) was presented for 6 minutes. During this time, *Scn1a*$^{RH/+}$ mice froze significantly more in response to the CS than the WT littermates ($t_{(22)}$=-3.700, p<0.01; **Fig. 4.3**B, Cue). In a closer examination of the time course of the cued fear conditioning task, it can be observed that the *Scn1a*$^{RH/+}$ mutants have a similar initial response to the tone as the WT littermates, but while the WT littermates show a steady decline in the amount of freezing over the six minutes of tone presentation, the freezing levels remain elevated in the *Scn1a*$^{RH/+}$ mutants (**Fig. 4.3**C). A two-way rANOVA analysis of the six-minute period of tone presentation reveals a significant main effect of genotype ($F_{(1,22)}$=13.687, p<0.01), a significant main effect of time ($F_{(6,132)}$=13.813, p<0.001), and a significant interaction between genotype and time ($F_{(6,132)}$=2.689, p<0.05). Post-hoc analysis (Tukey) shows a significant difference in freezing between *Scn1a*$^{RH/+}$ mutants and WT littermates during the last four minutes of the six-minute tone presentation (**Fig. 4.3**C). These data suggest that while the *Scn1a*$^{RH/+}$ mutants have a normal ability to form fear memories, they may have a deficit in their

ability to disassociate the learned connection between the CS and US and thus remained sensitized to the CS.

To measure sensorimotor gating, we used a prepulse inhibition (PPI) paradigm. There were no differences in the startle amplitude between *Scn1a*$^{RH/+}$ mutants and WT littermates to a startle stimulus (117 dB) presented alone (**Fig. 4.3**D). However, *Scn1a*$^{RH/+}$ mutants show deficits in the ability of a prepulse to inhibit the startle response (**Fig. 4.3**E). As the intensity of the prepulse increases, the amount of inhibition of the startle response should also increase. This was true for both genotypes as indicated by a main effect of PPI intensity ($F_{(3,66)}$=45.417, $p<0.001$) and no genotype-intensity interaction. However, there was also a main effect of genotype ($F_{(1,22)}$=5.475, $p<0.05$), and post-hoc analysis (Tukey) revealed that there was a significant reduction in PPI at a 76 dB prepulse intensity level between mutants and WT littermates. There was also a strong trend for a reduction in PPI at the 72 and 80 dB prepulse intensity levels. Overall, it appears that the *Scn1a*$^{RH/+}$ mutants have a deficit in the gating of sensory stimuli.

4.4.4 *Scn1a*$^{RH/+}$ mutants show a deficit in risk assessment ability

We originally chose the predator odor paradigm to measure anxiety and fear behavior in the *Scn1a*$^{RH/+}$ mutants. Mice were exposed to vehicle only on test day 1, to diluted predator odor 48-hours later, and then to vehicle only again after another 48-hour period. Two-way rANOVA analysis revealed a significant main effect of genotype ($F_{(1,18)}$=5.876, $p<0.05$), a significant main effect of odor exposure ($F_{(2,36)}$=26.614, $p<0.001$), and a significant interaction between genotype and odor exposure

($F_{(2,36)}$=4.110, p<0.05). Post-hoc analysis (Tukey) revealed that only the WT littermates froze significantly more to odor exposure than they did to the first vehicle exposure (**Fig. 4.4**A). While both genotypes exhibited contextual fear memory by freezing more on the second vehicle exposure in the same context as the predator odor exposure 48 hours earlier, the WT littermates again spent significantly more time freezing than the *Scn1a*$^{RH/+}$ mutants (**Fig. 4.4**A).

Since the *Scn1a*$^{RH/+}$ were freezing much less than WT littermates in response to the predator odor, we wanted to verify olfactory ability in these mice. In an odor discrimination task using an aversive odor, both *Scn1a*$^{RH/+}$ mutants and WT littermates spent less time in the side of the apparatus where the aversive odor was located (**Fig. 4.4**B) than would be expected by chance in a three-chambered apparatus (33.3%). To further verify olfactory ability, we also tested both groups with a positive odor (food). There was a main effect of food odor ($F_{(1,22)}$=14.280, p<0.01), and both *Scn1a*$^{RH/+}$ mutants and WT littermates spent more time in the chamber with the food than in the chamber without the food (**Fig. 4.4**C). Since none of the other behavioral tasks we subjected the *Scn1a*$^{RH/+}$ mutants to showed any measures of increased anxiety, and we had observed the *Scn1a*$^{RH/+}$ mutants charging headfirst into numerous situations without pausing to first assess the situation as WT animals would do (personal observations), we wondered if the mutants had an impaired ability to assess risk.

To answer this question, we used a virtual cliff avoidance paradigm. The testing apparatus has a transparent bottom and is set up to overhang a table top, creating the appearance of a sharp drop-off. The zone overhanging the table top was designated the "cliff" zone and the zone that sat safely on top of the table was designated the "safe"

zone. A narrow neutral zone separated the other two zones. A two-way ANOVA analysis revealed a main effect of zone ($F_{(2,44)}$=86.604, p<0.001) and a significant genotype-zone interaction ($F_{(2,44)}$=4.745, p<0.05), indicating that the two genotypes were not responding to the zones in the same manner. Post-hoc analysis (Tukey) revealed that, as expected, the WT littermates spent more time in the safe zone than in the cliff zone (**Fig. 4.4**D). However, the *Scn1a*$^{RH/+}$ mutants showed no discrimination between the safe zone and the cliff zone. After noticing that some animals were hugging the walls when venturing into the cliff zone and others went straight out over the apparent drop-off, we decided to compare the amount of time that animals spent in the middle of the cliff zone away from the walls. *Scn1a*$^{RH/+}$ mutants spent significantly more time in the open center of the cliff zone as compared to WT littermates ($t_{(22)}$=-2.774, p<0.05; **Fig. 4.4**E). This result suggested that either the *Scn1a*$^{RH/+}$ mutants could not see the virtual drop-off or they did not avoid it, possibly due to a lack of proper risk assessment ability. To ensure that an *Scn1a* mutation did not affect visual ability, we tested the visual acuity of both genotypes. There were no differences in visual acuity between *Scn1a*$^{RH/+}$ mutants and WT littermates (**Fig. 4.4**F), leading us to conclude that the *Scn1a*$^{RH/+}$ mutants have a deficit in the ability to assess risk. Additional risk assessment tasks were inconclusive (**Appendix B2**).

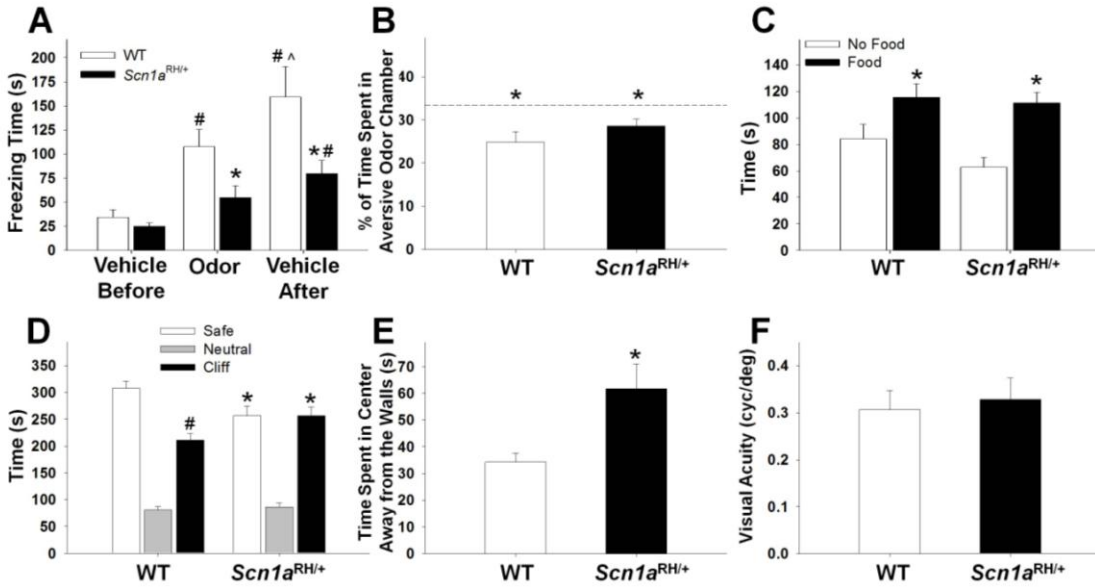

Figure 4.4 *Scn1a*$^{RH/+}$ mutants show deficits in risk assessment. (A) In response to predator odor exposure on test day 2, WT littermates, but not Scn1a$^{RH/+}$ mutants, froze significantly more than exposure to vehicle only 48-hours previously on test day 1. Both genotypes formed contextual fear memory, as they both froze more 48-hours later on the third test day to vehicle only, although the *Scn1a*$^{RH/+}$ mutants continued to freeze less than the WT littermates. *p<0.05 vs. WT within odor condition; #p<0.05 vs. Vehicle Before within genotype; ^p<0.05 vs. Odor within genotype. **(B)** The ability to detect an aversive odor was tested in a three-chambered apparatus. The percentage of time spent in the chamber containing the aversive odor was measured. A value of 33.3% represents chance (dotted line). Both *Scn1a*$^{RH/+}$ mutants and WT littermates spent significantly less than 33.3% of the time in the chamber with the aversive odor. *p<0.05 one-tailed t test vs. 33.3% chance. **(C)** The ability of *Scn1a*$^{RH/+}$ mutants to detect odor in general was tested using a novel food item. Both *Scn1a*$^{RH/+}$ mutants and WT littermates spent more time in the chamber containing the food item than in the chamber containing a control item. *p<0.05 vs. Control (no food). **(D)** During the visual cliff task, WT littermates spent less time on the side of the apparent cliff than on the safe side. However, *Scn1a*$^{RH/+}$ mutants do not differentiate the safe side from the cliff side. *p<0.05 vs. WT within zone; #p<0.05 vs. Safe Side within genotype. **(E)** A further examination of the visual cliff test reveals that when the mice are on the virtual cliff side of the apparatus, the WT littermates spend less time in the open center, away from the walls, than the *Scn1a*$^{RH/+}$ mutants. *p<0.05 vs. WT. **(F)** In an analysis of visual function, there is no difference between *Scn1a*$^{RH/+}$ mutants and WT littermates in measures of visual acuity. n=10-12 all groups (A-E), n=5 both groups (F). Error bars represent SEM.

4.4.5 *Scn1a*$^{RH/+}$ mutants show mild social deficits

In a three-chambered social task where mice had a choice between a stranger mouse and an empty cage (an inanimate object), both *Scn1a*$^{RH/+}$ mutants and WT littermates showed a preference for a stranger mouse over the empty cage (**Fig. 4.5**A). A two-way rANOVA showed only a significant main effect of the stranger mouse ($F_{(1,22)}$=42.524, p<0.001). There was no significant main effect of genotype nor interaction between genotype and stranger. The second task involved a choice between an already familiar mouse and a novel mouse. Only the WT littermates showed a significant preference for the novel mouse over the familiar mouse (**Fig. 4.5**B). Again, there was a main effect of the stranger mouse ($F_{(1,20)}$=10.707, p<0.01), but post-hoc analysis revealed that only the WT littermates had a significant preference for the novel mouse. Although they are exhibiting some level of social behavior, this result would indicate that the *Scn1a*$^{RH/+}$ mutants have a mild deficit in their preference for social novelty.

During a mating call analysis paradigm, all males readily investigated the unfamiliar female mouse upon her introduction to the cage, and vocalizations were detected in all recordings. In an analysis of the first four minutes of the exposure to the female, there was a strong trend for a main effect of genotype ($F_{(1,21)}$=4.211, p=0.053) and a significant main effect of time ($F_{(3,63)}$=48.417, p<0.001) indicating that the number of calls decreased as interaction time increased. Post-hoc analysis revealed a significant difference in calls during the first minute, with WT littermates emitting more calls than the *Scn1a*$^{RH/+}$ mutants (**Fig. 4.5**C). After the first four minutes of the test had elapsed, the call numbers had decreased dramatically and there were no longer any genotype

differences (data not shown). Moreover, there were no significant genotype differences in mean call duration or mean call frequency (data not shown). Overall, during the first four minutes of interaction time, *Scn1a*$^{RH/+}$ mutants were emitting fewer calls than their WT littermates.

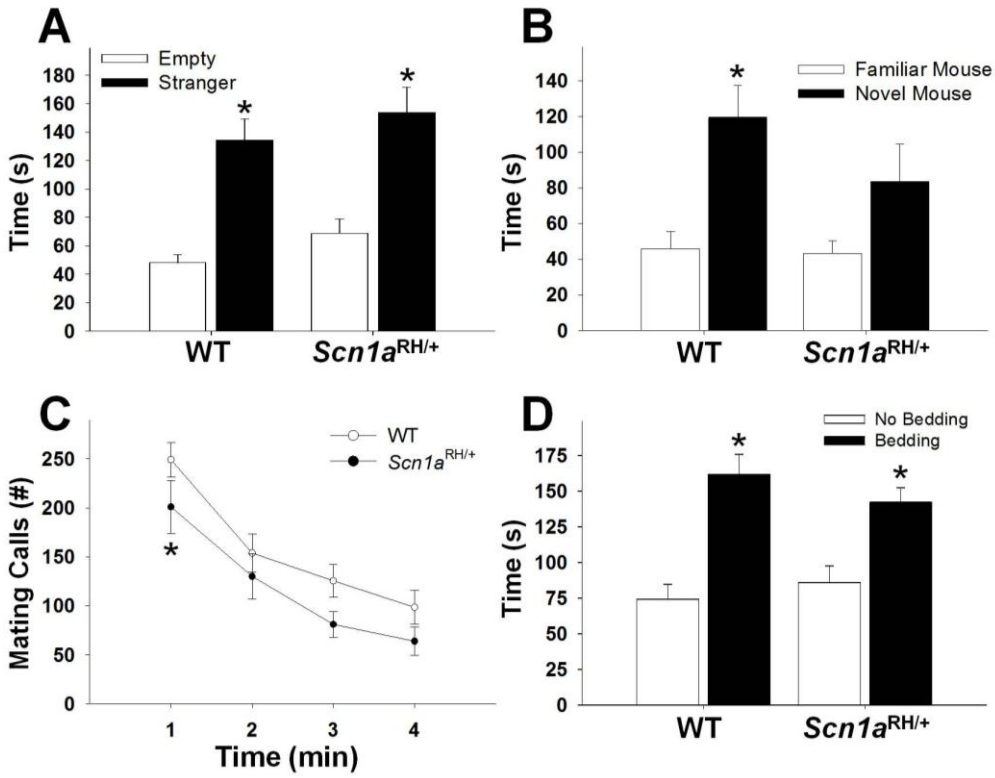

Figure 4.5 *Scn1a*$^{RH/+}$ mutants show mild social deficits. (A) In a choice between a chamber with an empty cage and a chamber with a cage housing a stranger mouse, both *Scn1a*$^{RH/+}$ mutants and WT littermates prefer to spend time with the stranger mouse. *p<0.05 vs. Empty Chamber. **(B)** In a choice between a familiar mouse and a novel mouse, only the WT littermates show a significant preference for the novel mouse. *p<0.05 vs. Familiar Mouse. **(C)** When exposed to an unfamiliar female mouse, male mice will exhibit numerous mating calls. In the first minute of exposure, WT littermates emit more calls than the *Scn1a*$^{RH/+}$ mutants, and an overall main effect of genotype indicates that WT littermates are calling more in general than *Scn1a*$^{RH/+}$ mutants. *p<0.05 vs. WT. **(D)** In an olfactory discrimination test using a socially relevant odor (soiled bedding), both *Scn1a*$^{RH/+}$ mutants and WT littermates showed a preference for the chamber containing the social odor. *p<0.05 vs. No Bedding. n=12 all groups. Error bars represent SEM.

As part of a series of olfactory discrimination tasks, we tested the ability of *Scn1a*$^{RH/+}$ mutants to detect a socially relevant odor by placing soiled bedding from an unfamiliar male mouse into one chamber of the three-chambered apparatus. Both *Scn1a*$^{RH/+}$ mutants and WT littermates spent more time in the chamber containing the

bedding (**Fig. 4.5**D). There was a main effect of bedding odor ($F_{(1,22)}$=31.129, p<0.001) but no main effect of genotype nor genotype-odor interaction. This indicates that the *Scn1a*$^{RH/+}$ are responsive to socially relevant odors. Although the *Scn1a*$^{RH/+}$ mutants have a normal preference for socialability and a socially relevant odor, the reduced response to social novelty and the reduced number of mating calls suggests that the *Scn1a*$^{RH/+}$ have a mild social impairment.

4.4.6 Stress worsens seizure outcome in *Scn1a*$^{RH/+}$ mutants

We first analyzed the effects of acute restraint stress and chronic daily restraint stress on spontaneous seizure activity. As previously noted, only two of the eight animals analyzed with EEG recordings showed spontaneous seizures during the baseline recording period. Following chronic daily restraint stress (20-minute restraint once a day for six days), the two previously seizing animals did begin to have daily, more frequent seizures, described as follows:. Mouse #1 had an average of one seizure per day during baseline recordings yet exhibited between two to five seizures per day during and following chronic stress administration. Mouse #2 had only one seizure during the baseline recording period but progressed to one seizure per day during and following chronic stress administration. The six non-seizing animals did not show any seizure activity following an acute or chronic stressor. It is known that family members with the same *SCN1A* mutation often exhibit a wide range of epilepsy subtypes and severities (Scheffer and Berkovic 1997), and in light of our spontaneous seizure results, it is interesting to speculate that stress may only be impacting those that are already exhibiting

a seizure phenotype and is not sufficient to initiate seizure activity in those not already experiencing seizures.

We next tested the effects of acute restraint stress on picrotoxin-induced seizure thresholds. There were no differences in the latencies to a myoclonic jerk, the first stage of a picrotoxin-induced seizure (data not shown). In analysis of the latency to the second stage (atypical clonic seizure), we found a main effect of restraint stress ($F_{(1,38)}$=9.317, $p<0.01$). Post-hoc analysis revealed that there was a statistically significant decrease in the latencies of the *Scn1a*$^{RH/+}$ mutants, but not in the WT littermates (**Fig. 4.6**A). The third stage of a picrotoxin-induced seizure is the full development of bilateral forelimb clonus. Again we found a main effect of restraint stress ($F_{(1,39)}$=12.094, $p<0.01$), but this time post-hoc analysis showed significant decreases in both the *Scn1a*$^{RH/+}$ mutants and the WT littermates (**Fig. 4.6**B). An analysis of the latency to a stage 4 picrotoxin-induced seizures, the GTCS without a tonic component, again revealed a main effect of restraint stress ($F_{(1,39)}$=8.719, $p<0.01$); however, the post-hoc analysis showed that the decrease in threshold was significant only in the *Scn1a*$^{RH/+}$ mutants (**Fig. 4.6**C). Interestingly, there were no differences in latencies to the Stage 5 picrotoxin-induced seizure (GTCS with tonic hindlimb extension); however, it was observed that the *Scn1a*$^{RH/+}$ mutants were having many more repeated Stage 4 seizures prior to advancing to a Stage 5 seizure (WT littermates, 2.8 ± 0.8 Stage 4 Seizures; *Scn1a*$^{RH/+}$ mutants, 6.5 ± 1.5 Stage 4 Seizures; $t_{(19)}$=-2.05, p=0.054). While stress was sufficient to decreased seizure thresholds in both genotypes, the decreases seen in the *Scn1a*$^{RH/+}$ mutants were more pronounced.

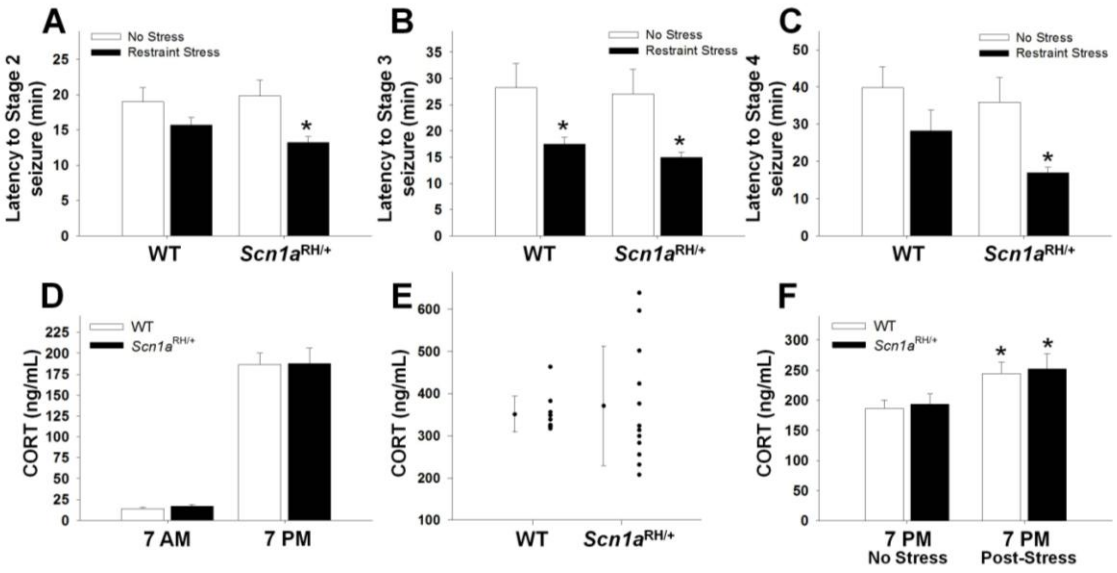

Figure 4.6 Stress Worsens Seizures in *Scn1a*$^{RH/+}$ Mutants. (A) A 20-minute acute restraint stress significantly reduced the latency to a Stage 2 picrotoxin-induced seizure (atypical clonic seizure) in *Scn1a*$^{RH/+}$ mutants, but not in WT littermates. *p<0.05 vs. No Stress. **(B)** Stress significantly decreased latency to a Stage 3 picrotoxin-induced seizure (bilateral forelimb clonus) in both *Scn1a*$^{RH/+}$ mutants and WT littermates. *p<0.05 vs. No Stress. **(C)** Stress significantly decreases the latency to a Stage 4 picrotoxin-induced seizure (GTCS) in *Scn1a*$^{RH/+}$ mutants while the post-hoc results were not significant in WT littermates. *p<0.05 vs. No Stress. **(D)** *Scn1a*$^{RH/+}$ mutants have normal diurnal fluctuations in plasma corticosterone levels, with similar levels as WT littermates during both the nadir (7 am) and peak (7 pm) of the circadian HPA axis rhythm. **(E)** Although the average corticosterone response to an acute 20-minute restraint stress is not significantly different between genotypes, the *Scn1a*$^{RH/+}$ mutants have a wider range of variability in their corticosterone response. **(F)** Morning exposure to acute restraint stress increased corticosterone levels at 7 pm in both *Scn1a*$^{RH/+}$ mutants and WT littermates. *p<0.05 vs. 7 pm No Stress. n= 12 all groups. Error bars represent SEM.

4.4.7 *Scn1a*$^{RH/+}$ mutants have similar HPA axis function as WT littermates

To determine if a mutation in *Scn1a* could affect the function of the HPA axis, we examined basal corticosterone (CORT) levels at both the nadir (7 am) and peak (7 pm) of the diurnal CORT rhythm. There were no differences between genotypes in basal CORT levels (**Fig. 4.6**D). Next, we examined the CORT response to an acute 20-minute restraint stress. Again, there were no genotype differences, although we did note that the *Scn1a*$^{RH/+}$ mutants had a greater variation in their CORT response (**Fig. 4.6**E), perhaps recapitulating the wide range of phenotypes seen in human patients with *SCN1A* mutations. Finally, we examined CORT at 7 PM on the same day that the mice were exposed to acute restraint stress in the morning (8-9 am), CORT levels in both *Scn1a*$^{RH/+}$ mutants and WT littermates were elevated as compared to normal, non-stress 7 PM CORT levels (**Fig. 4.6**F). A two-way rANOVA revealed a main effect of stress ($F_{(1,20)}$=9.294, $p<0.01$). The lack of significant differences between *Scn1a*$^{RH/+}$ mutants and WT littermates suggests that the *Scn1a* mutation is not affecting normal HPA axis function.

4.4.8 *Scn1a*$^{RH/+}$ mutants do not show any depressive-like behaviors

The *Scn1a*$^{RH/+}$ mutants did not show any deficits in experiments measuring depressive-like behaviors. For details on experiments and data, see **Appendix B3**.

4.5 Discussion

We had previously reported that homozygous *Scn1a*$^{RH/RH}$ mutants developed spontaneous seizures and weight loss starting at P15 (Martin et al. 2010), the time when *Scn1a* expression approaches adult levels (Ogiwara et al. 2007). We also reported an average life span of 18.5 days with 100% mortality by P26 (Martin et al. 2010). These previous studies were conducted at the N2 generation on a mixed 129X1/SvJ x C57BL/6J background. Because the genetic background is known to affect seizure phenotypes, behavior phenotypes, and stress responsivity (Anisman et al. 2001; Schauwecker 2002; Moy et al. 2004; Brinks et al. 2008), we decided to backcross the *Scn1a* R1648H line to the C57BL/6J background to obtain the more congenic N12 generation. The N12 homozygous *Scn1a*$^{RH/RH}$ mutants were observed to have spontaneous seizures beginning on P10. While weight loss was not observed until P16, we noted a significant slowing in the growth rate beginning at P10, at a time when *Scn1a* levels are beginning to noticeably increase (Ogiwara et al. 2007). The average life span of *Scn1a*$^{RH/RH}$ mutants in this study was 24.5 days, with 9 out of 38 homozygous mutants living past P26. While the majority of homozygous mutants had died by P26, we did have one mouse that survived to P88. The relatively long-lived homozygous mutants did initially show weight loss around P16, although after weaning (P21) they began to show slight weight gains; however, they remained small and never weighed more than 13 g. The premature lethality seen in the homozygous *Scn1a*$^{RH/RH}$ mutants may explain why homozygous mutations have never been reported in GEFS+ patients.

We previously reported that heterozygous *Scn1a*$^{RH/+}$ mutants had a normal life span, infrequent spontaneous seizures, and a reduced latency to flurothyl-induced

seizures (Martin et al. 2010). The N12 *Scn1a*$^{RH/+}$ mutants also show infrequent spontaneous seizures and a reduced latency to flurothyl-induced seizures, but, interestingly, we observed a significant difference in the life span of *Scn1a*$^{RH/+}$ mutants as compared to WT littermates. At the end of the 90-day experimental timeframe, only 83% of the *Scn1a*$^{RH/+}$ mutants were still alive. While this still represents a majority of mutants surviving, the difference was significant when compared to the 100% survival rate of WT littermates at P90. The difference in survival of the N2 and N12 heterozygous mutants is likely due to differences in the genetic background. In their study of a nonsense *Scn1a* mutation, Ogiwara et al. (2007) show that heterozygous mutants had 100% survivability on a mixed 129X1/SvJ x C57BL/6J (75%/25%) background but reduced survivability on a background weighted more toward the C57BL/6J strain (75%). Other studies with mouse models of DS have also demonstrated a more severe phenotype on the C57BL/6J background (Yu *et al.* 2006; Miller *et al.* 2013). While GEFS+, unlike DS, is typically not associated with high mortality, there have been cases of sudden unexpected death (SUDEP) in at least one GEFS+ family (Hindocha et al. 2008). In light of the role of background strain on lethality in the *Scn1a*$^{RH/+}$ mutants, it is possible that additional modifier genes may influence survival in individuals with *SCN1A* mutations.

Further findings in this study confirm the proposed link between *Scn1a* dysfunction and autistic-like behaviors. Recently, two studies have shown that mice with *Scn1a* haploinsufficiency exhibit hyperactivity, profound social deficits, anxiety, and impaired spatial learning and memory (Han et al. 2012; Ito et al. 2012). While the more severe haploinsufficiency of a *Scn1a* knockout recapitulates the phenotype of DS, the neuropsychiatric comorbidities and cognitive deficits in GEFS+ have not previously been

studied in an animal model. In this study, we have shown that the *Scn1a*$^{RH/+}$ mutants also demonstrate hyperactivity, social deficits, and impaired learning and memory. As the seizure phenotype of GEFS+ is less severe than that of DS, it was not surprising for us to find milder behavioral phenotypes in the GEFS+ mice. While levels of hyperactivity were comparable, the *Scn1a*$^{RH/+}$ mutants exhibited milder social deficits. While mice with *Scn1a* haploinsufficiency showed profound social deficits, the *Scn1a*$^{RH/+}$ mutants showed normal social preference for both social contact and social odor. However, the *Scn1a*$^{RH/+}$ mutants did show deficits in preference for social novelty as well as reduced mating calls. While there has been widespread evidence linking ASDs with DS, fewer studies have published results of autistic-like symptoms in GEFS+ patients. In two studies that have linked GEFS+ with autism, the mutation in one is linked with a homozygous *Scn1a* mutation (Tan et al. 2012) and the mutation in the other does not involve VGSCs (Dixon-Salazar et al. 2004). Nevertheless, the findings of the current study suggest that the autistic-like traits in GEFS+ may be milder in comparison to those seen in DS, and perhaps more likely to be overlooked in clinical presentations.

Both Han et al. (2012) and Ito et al. (2012) reported deficits in spatial learning ability in mice with *Scn1a* haploinsufficiency. Likewise, the *Scn1a*$^{RH/+}$ mutants showed deficits in spatial object recognition in this study. Our lab recently showed that 91% of the parvalbumin interneurons in the CA1, CA3, and DG regions of the hippocampus stained positive for *Scn1a* (Dutton et al. 2012), and the hippocampus is known to be an important region for spatial learning (Foster and Knierim 2012). Interestingly, while the *Scn1a*$^{RH/+}$ mutants did not show deficits in contextual fear learning, also a hippocampus-dependent task, they did show enhanced discrete (cued) fear learning, a task that also

involves the amygdala (Phillips and LeDoux 1992). Interestingly, a recent novel finding by Singer et al. (2013) was that habituation to a startle stimulus was correlated with spatial reference memory. Mice that showed a strong habituation to a repeated startle stimulus out-performed those that showed sensitization to the startle stimulus in a water maze reference memory test. Similarly, in the present study, the *Scn1a*$^{RH/+}$ mutants show reduced spatial recognition memory and appear to remain sensitized to the tone in the cued fear conditioning task. As this link between sensitization and spatial reference memory is new to the field, the underlying neural mechanisms that could be connecting these two behaviors have not yet been explored. While dopaminergic and glutamatergic mechanisms have been suggested (Singer et al. 2013), the results of this study suggest that inhibitory networks are also likely playing a role in this connection.

Another novel finding of this study is the reduction in PPI and increase in risk-taking behavior in the *Scn1a*$^{RH/+}$ mutants. As neither of these phenotypes has previously been described in *Scn1a* mutants, these findings further extend the spectrum of phenotypes associated with *Scn1a* dysfunction. PPI of the acoustic startle response involves a pre-attentive gating mechanism of incoming sensory stimuli, also known as sensorimotor gating. While deficits in PPI have been strongly linked with schizophrenia, the connection between PPI deficits and autism is poor and no evidence links altered PPI with attention deficit/hyperactivity disorder (ADHD) (Kohl et al. 2013). Intriguingly, it was recently reported that WAG-Rij rats, a model of genetic absence epilepsy, also have deficits in PPI which were reversible by lamotrigine (Celikyurt et al. 2012), an antiepileptic medication that works to block VGSCs but that can also increase GABAergic neurotransmission (Cunningham and Jones 2000). In another study, kindling

of the prefrontal cortex resulted in both a PPI deficit and hyperactivity (Ma and Leung 2010), two of the phenotypes seen in the *Scn1a*$^{RH/+}$ mutants. Given that *Scn1a* expression is high in the prefrontal cortex (Dutton et al. 2012), this region may be mediating both the PPI and hyperactivity phenotypes seen in the *Scn1a*$^{RH/+}$ mutants. In fact, Shamir et al. (2012) showed that alterations in parvalbumin interneurons was correlated with hyperactivity, reductions in PPI, and deficits in cued fear conditioning, suggesting that the dysfunction of *Scn1a* in this subset of interneurons could be driving these phenotypes. To date, the only form of epilepsy linked to deficits in PPI is temporal lobe epilepsy with psychosis (Braff et al. 2001), although it is unclear to what extent clinical testing of patients with *SCN1A* mutations for PPI deficits has been done.

While it is known that some behavioral problems and comorbid neuropsychiatric abnormalities exist in patients with epilepsy, risk-taking behavior in this population has been poorly studied. A relatively recent study by Alfstad et al. (2011) has tried to fill in this gap by studying risk-taking behavior in young persons with epilepsy. This study found that risk-taking behaviors were more prevalent in young boys with epilepsy than in the controls. Given that risk-taking behavior in humans has been associated with prefrontal cortex activity (Studer et al. 2013), and prefrontal cortex activity has also been linked with hyperactivity, PPI, and cued fear conditioning as discussed above, it would not be surprising to find an increase in risk-taking behavior in our *Scn1a*$^{RH/+}$ mutants. Indeed, after observing that the *Scn1a*$^{RH/+}$ mutants froze less than WT littermates when exposed to predator odor and also that *Scn1a*$^{RH/+}$ mutants would proceed into a new situation without first pausing to evaluate the danger as the WT littermates were observed to do (personal observations), we began to wonder if the *Scn1a*$^{RH/+}$ mutants might have a

deficit in their ability to properly assess risk in dangerous situations. Our findings in the visual cliff task would seem to support this hypothesis. In fact, an increase in risk-taking behavior could be related to hyperactivity disorder as ADHD patients show high levels of impulsivity (Ibanez et al. 2012). This intriguing new phenotype in the *Scn1a*$^{RH/+}$ mutants highlights the importance of *Scn1a* expression in the prefrontal cortex and opens up new avenues of investigation into comorbidities associated with *Scn1a* dysfunction.

Much of the previous work investigating the role of stress as a seizure trigger has been done in wildtype animals with normal brain and stress system development (Sawyer and Escayg 2010), yet our lab has recently shown that a mutation in another VGSC, *Scn8a*, can alter the seizure response to a stressor (Sawyer *et al.* 2014). In this current study, we found that acute restraint stress did reduce thresholds to picrotoxin-induced seizures. Although the direction of change was the same in both WT littermates and *Scn1a*$^{RH/+}$ mutants, the magnitude of the change was more significant in the *Scn1a*$^{RH/+}$ mutants. Furthermore, *Scn1a* dysfunction does not seem to affect normal functioning of the HPA axis, one of the primary physiological stress response systems. While these results differ from the ones found with the *Scn8a* mutants, the differences shed light on possible pathophysiological mechanisms. *Scn8a* mutants have deficits in excitatory signaling, spontaneous absence seizures, and an increased resistance to chemiconvulsant-induced seizures (Martin et al. 2007; Papale et al. 2009). Furthermore, the *Scn8a* mutants showed measures of increased anxiety and it was suggested that the increased anxiety was triggering long-term changes in HPA axis activity as well as altering the seizure response to stress (Sawyer *et al.* 2014). The *Scn1a*$^{RH/+}$ mutants did not show any differences in measures of anxiety tested in this study, and perhaps for this reason the

effect of stress on the response to picrotoxin-induced seizures is similar between *Scn1a*$^{RH/+}$ mutants and WT littermates. Interestingly, the chronic restraint stress only increased spontaneous seizure activity in the *Scn1a*$^{RH/+}$ mutants who were already exhibiting spontaneous seizures, suggesting that while stress can precipitate spontaneous seizures in an already seizing animal, it may not be sufficient to cause seizures in a mutant that does not have a history of spontaneous seizures.

In summary, we have shown that the *Scn1a*$^{RH/+}$ phenotype is more severe on a congenic C57BL/6J background, suggesting that other modifier genes could contribute to the clinical heterogeneity seen in GEFS+ families. We also verified autistic-like traits such as hyperactivity and social deficits as well as deficits in spatial memory that have previously been linked with *SCN1A* dysfunction. However, this study is the first to extend the *Scn1a* phenotype to include deficits in cued fear conditioning, PPI, and risk assessment which provides additional insight into the pathophysiological mechanisms underlying a VGSC dysfunction. Finally, we have shown that stress can worsen seizure outcome in *Scn1a*$^{RH/+}$ mutants in a similar manner as seen in WT littermates, and the *Scn1a* mutation does not affect normal HPA axis function.

4.6 Acknowledgements

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CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

5.1 Summary

With this body of work, we have demonstrated that a genetic predisposition to epilepsy, and mutations in voltage-gated sodium channels (VGSCs) more specifically, can affect the stress response, seizure outcome following a stressor or altered early life experience, and behavior. The results described herein provide a better understanding of how stress and other environmental factors can interact with altered brain excitability to affect seizure outcome and behavior. The *Scn8a*$^{med/+}$ mutant exhibits two distinct seizure phenotypes: a increased threshold to exogenously induced seizures and spontaneous spike-wave discharges (SWDs) characteristic of absence epilepsy (Schaller et al. 1995; Martin et al. 2007; Papale et al. 2009). The *Scn1a*$^{RH/+}$ mutant recapitulates the phenotype of genetic epilepsy with febrile seizures plus (GEFS+), a human epilepsy condition characterized by febrile seizures that persist beyond six years of age and the development of afebrile generalized or partial seizures during adulthood (Scheffer and Berkovic 1997). We have shown that the *Scn8a*$^{med/+}$ mutation affects the stress response, the seizure response to stress, behavior, and adult outcome following early life enrichment. On the other hand, the *Scn1a*$^{RH/+}$ mutation does not appear to affect the stress response nor the seizure response to stress, but it does have a large impact on behavior. In the following sections we will discuss in more detail the results described herein and suggest future directions for this work.

5.2 VGSCs and the Response to Acute Stress

The response to acute stress involves activation of the hypothalamic-pituitary-adrenal (HPA) axis, which begins with neurons in the paraventricular nucleus (PVN) of the hypothalamus releasing corticotropin-releasing hormone (CRH). CRH stimulates the pituitary to release adrenocorticotropic hormone (ACTH) into the bloodstream, where it acts on the adrenal cortex to cause release of glucocorticoids (cortisol in humans and corticosterone (CORT) in rodents). The glucocorticoids mobilize energy stores, suppress the immune response, and work as mediators of a negative feedback loop that dampens activity of the HPA axis. It is also clear that the HPA axis is regulated by input from many brain regions, including the prefrontal cortex, hippocampus, amygdala, and the bed nucleus of the stria terminalis (BNST) (Herman et al. 2003). Recent evidence suggests that control of the HPA axis by central regions is largely under GABAergic control (Sarkar et al. 2011), suggesting that alterations in the balance of brain excitability could affect HPA axis function.

In wildtype animal studies, acute stress has been shown to be largely anti-convulsant, although results differ depending on the models and stressors used (Sawyer and Escayg 2010). In the experiments described in this dissertation, the acute stressor used was a 20-minute restraint stress. We chose restraint stress because it is considered to be a processive stressor, a stressor that involves higher-order sensory processing and limbic pathways (Anisman et al. 2001). Furthermore, restraint stress can also be classified as a neurogenic stressor, a stress that is purely psychological (Anisman et al. 2001). We chose this type of stressor in an attempt to mimic emotional stressors, as this is

the type of stressor reported by epilepsy patients as most apt to trigger a seizure (Nakken et al. 2005).

5.2.1 Short-Term Effects of Acute Stress on Spontaneous Seizures

We found that an acute stressor was able to trigger an immediate increase in SWDs in *Scn8a*$^{med/+}$ mutants, between 40 and 80 minutes after the onset of the stressor. While spontaneous seizure in the *Scn1a*$^{RH/+}$ mutants have been observed during routine colony procedures such as cage changing (personal observation), we did not detect any spontaneous seizures in response to acute restraint stress. Interestingly, neither mutant showed a difference in the CORT response to the acute stressor, although the variability of the CORT response in the *Scn1a*$^{RH/+}$ was extremely high, possibly depending on the frequency of seizure activity at baseline within each individual. This suggests that these two VGSC mutations may not be altering the normal response of the HPA axis to an acute stressor. While other genetic models of epilepsy have been shown to exhibit altered HPA axis activity, these responses vary from model to model. The EL mouse shows normal basal levels of CORT but a sustained high CORT response after an acute stressor (Forcelli et al. 2007), while the WAG/Rij rats show elevated basal levels of CORT and a more rapid normalization of CORT after acute stress (Tolmacheva et al. 2012). Our lab has previously shown that another *Scn8a* mutant, *Scn8a*$^{med\text{-}jo}$, has a flattened diurnal CORT profile (Papale et al. 2010). However, the *Scn8a*$^{med\text{-}jo}$ mutation is a point mutation resulting in altered biophysical properties of $Na_v1.6$ channels, while the *Scn8a*^{med} mutation is a more severe null mutation. Furthermore, the *Scn8a*$^{med\text{-}jo}$ line is maintained

on the more stress reactive C57BL/6J background, while the C3HeB/FeJ line is considered to be less stress-reactive (Belzung et al. 2001). It appears that the influence of an epileptic brain on the HPA axis is dependent on a number of factors, including the nature of the altered excitability, modifier genes, and possibly the frequency of baseline seizure activity.

In the *Scn8a*$^{med/+}$ mutant, the elevation in CORT levels following the acute stress preceded the short-term increase in spontaneous SWD activity. Further evidence that CORT may be driving the short-term increase in seizures comes from a study with WAG/Rij rats in which direct injections of CORT increased SWD activity 15-30 minutes after injection (Schridde and van Luijtelaar 2004). It has previously been shown that corticosterone can directly alter sodium currents (Werkman et al. 1997) and sodium conductances (Joels 1997), thus CORT can exert pro-convulsive effects by altering excitability and network activity. In the brain, CORT acts through mineralocorticoid receptors (MRs) and glucocorticoids receptors (GRs), and these two receptors are differentially distributed within the brain (De Kloet et al. 2005). MRs are highly expressed in the hippocampus and lateral septum and moderately expressed in the amygdala, the PVN of the hypothalamus, and the locus coeruleus (De Kloet et al. 2005). GRs are ubiquitously expressed in the brain, but enriched in the hippocampus, lateral septum and the PVN (De Kloet et al. 2005). In general, CORT increases neuronal excitability through rapid action at MRs and reduces excitability through slower action at GRs (Joels 2009), although it has also been shown that CORT can occasionally increase excitability through GR activation in specific areas of the brain such as the basolateral

amygdala (Duvarci and Pare 2007). Thus, one possible mechanism by which stress could be affecting seizure activity is through the actions of CORT.

However, CORT may not be the only mediator of the HPA axis that could be driving increased seizure activity following an acute stress. CRH has also been shown to have pro-convulsive effects. Intracerebroventricular injections of CRH can induce seizure activity (Ehlers et al. 1983; Weiss et al. 1986; Baram and Schultz 1991), with the seizure activity initiating in limbic areas such as the hippocampus and amygdala (Marrosu et al. 1988; Baram et al. 1992). Given the role of the limbic system in emotional reactivity and the fact that emotional stress is a potent seizure trigger, CRH may well be a key player in the stress-induced seizure response. Rats deficient in CRH are resistant to electrical kindling (Weiss et al. 1993), and the highly seizure-susceptible EL mouse shows higher baseline CRH immunoreactivity (Forcelli et al. 2007). In fact, impairment of the CRH system in EL mice can reduce handling-induced seizure susceptibility (Pascual and Heinrichs 2007). Thus, multiple mediators within the HPA axis response to an acute stress may be driving the increase in spontaneous seizure activity seen in the *Scn8a*$^{med/+}$ mutants. Further work is therefore needed to clarify the role of these HPA axis mediators in the increase of spontaneous SWD activity in response to an acute stressor (see Future Directions).

5.2.2 Short-Term Effects of Acute Stress on Induced Seizures

The artificial induction of seizures in the *Scn8a*$^{med/+}$ mutants and *Scn1a*$^{RH/+}$ mutants yielded interesting results. In our first experiments with the *Scn8a*^{med} line, an acute 20-minute restraint stress proved to be anti-convulsant in the WT littermates, confirming what had been previously reported in the literature with WT rodents. In contrast, stress decreased thresholds to picrotoxin-induced seizures in the *Scn8a*$^{med/+}$ mutants and increased seizure severity, suggesting that the *Scn8a* mutation was sufficient to alter the response to the acute stress. We repeated the experiment with the *Scn1a*^{RH} line to see if the results could be generalized to a different VGSC. While the acute stress also decreased thresholds to picrotoxin-induced seizures in the *Scn1a*$^{RH/+}$ mutants, it also reduced thresholds in the WT littermates, in contrast to our previous results with the *Scn8a*^{med} line. As discussed later in the "VGSCs and Behavior" section, the *Scn8a*$^{med/+}$ mutation increases anxiety levels, while the *Scn1a*$^{RH/+}$ mutation does not. Also, the C57BL/6J background of the *Scn1a*^{RH} line is considered to be more stress reactive than the C3HeB/FeJ background of the *Scn8a*^{med} line (Belzung et al. 2001; van Bogaert et al. 2006). While more reactive to stress, anxiety levels in C57BL/6J mice are considered to be low (Rodgers et al. 2002). We propose that it is the elevated anxiety seen in the *Scn8a*$^{med/+}$ mutants, but not the WT C3HFeB/FeJ littermates, that interacts with the acute stress and that is responsible for the decreased seizure thresholds seen in the *Scn8a*$^{med/+}$ mutants (**Fig. 5.1**). On the other hand, the increased reactivity of the C57BL/6J background strain might be responsible for reducing seizure thresholds in both the *Scn1a*$^{RH/+}$ mutants and the WT C57BL/6J littermates (**Fig. 5.1**). In the first case, the

VGSC mutation (*Scn8a*) is the mediator in the stress-seizure interaction, whereas in the second case, the VGSC mutation is not a direct player in the stress-seizure interaction. These results would suggest that *Scn8a* plays a larger role in pathways responsible for connecting the stress response and seizure generation, and that these pathways may overlap with or are part of the same pathways that drive anxiety-like behaviors.

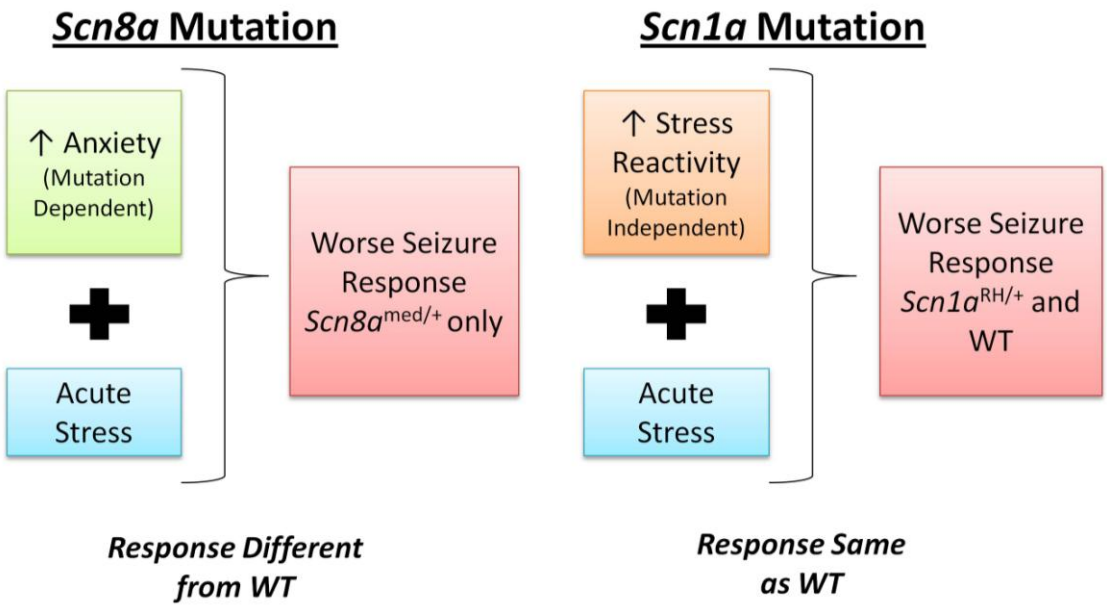

Figure 5.1 Proposed Model of VGSC Interaction with Acute Stress. In the literature, increased seizure threshold is the normal response to acute stress. In *Scn8a*$^{med/+}$ mutants, acute stress worsens seizure outcome while it is anti-convulsant in the WT littermates as expected. These different responses could be caused by the increase in anxiety-like behaviors seen in only the *Scn8a*$^{med/+}$ mutants. In the *Scn1a*$^{RH/+}$ mutants, acute stress worsens seizure outcome in both the mutants and the WT littermates. This similarity in a pro-convulsant response could be due to the increased stress reactivity that is common to the background strain (C57BL/6J) of all animals in this line.

In the literature, acute stress has been shown several times to be anti-convulsant in WT mice (De Lima and Rae 1991; Pericic and Bujas 1997; Pericic et al. 2000; Pericic et al. 2001). Interestingly, none of these experiments have been done on the C57BL/6J

background. In our studies, acute stress was also anti-convulsant in the WT C3HeB/FeJ mice as would be expected from the literature. Interestingly, in a preliminary study we did with an earlier generation (N2) of the *Scn1a*^{RH} line, acute stress was also anti-convulsant in WT littermates on a mixed 129X1/SvJ x C57BL/6J background (**Fig. 5.2**). Once the strain was advance to N12 (on the more congenic C57BL/6J background), acute stress became pro-convulsant in the WT mice, suggesting that the C57BL/6J background itself contains modifier genes that worsen the seizure response to stress. This supposition is strengthened by another study in which a GABAergic compound did not produce as large of an anticonvulsant effect in the C57BL/6J strain as compared to the BALB/cByJ strain (Verleye et al. 2011). A recent genetic study attempted to determine which loci were involved in the increased stress reactivity of C57BL/6J mice as compared to a C3H/HeJ strain. They found a significant candidate gene, *Enoph1*, which codes for enolase phosphatase 1, an important protein in the methionine salvage pathway (Barth et al. 2013). C57BL/6J mice have significantly lower levels of *Enoph1* in the cingulated cortex, hippocampus, and hypothalamus as compared to C3H/HeJ mice, and exposure to stress increased expression of *Enoph1* (Barth et al. 2013). Intriguingly, the loci identified was an area of mouse chromosome 5 which is homologous to a region on human chromosome 4 that has been associated with agoraphobia and panic disorder (Barth et al. 2013). While more research is needed to determine the precise genes and pathways involved, it is clear from the studies presented herein that the C57BL/6J background is sufficient to worsen seizure outcome following exposure to acute stress.

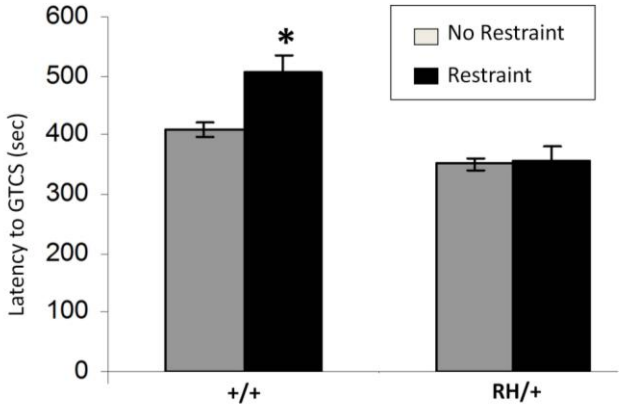

Figure 5.2 Heterozygous N2 *Scn1a*$^{RH/+}$ mutants differ from wild-type littermates in their response to stress. Latencies to flurothyl-induced generalized tonic-clonic seizures (GTCS) were measured in both males and females. No sex differences were observed, so data were combined. Differences in latencies to flurothyl-induced GTCS between non-restrained (n = 28) and restrained (n = 27) wild-type animals were statistically significant (no restraint, 410 ± 11s; restraint, 506 ± 30s, p = 0.001). Latencies to GTCS between non-restrained (n = 31) and restrained (n = 26) mutants were not statistically different (no restraint, 351 ± 9s; restraint, 358 ± 23s, p = 0.787). +/+, Wild-type; RH/+, heterozygous R1648H. *, p = 0.001. Error bars represent SEM.

5.2.3 Long-Term Effects of Acute Stress

Interestingly, although seizure frequency returned to baseline levels within two hours following the restraint in *Scn8a*$^{med/+}$ mutants, acute stress also resulted in a broadening in the evening peak of SWD that normally occurs at the beginning of the dark period. This change in the circadian rhythm of seizures persisted for up to 60 hours after the initial acute stress. Circadian variation in seizure activity has long been recognized, and it has been reported that patients with epilepsy have one or more "time peaks" in seizure activity, although these time peaks vary from group to group (Langdon-Down and Brain 1929; Kellaway et al. 1980; Hofstra and de Weerd 2009). Furthermore, a circadian rhythm of SWDs similar to the one described in our studies was recently described in another model of absence epilepsy, the WAG/Rij rats (Smyk et al. 2011). Smyk et al.

(2011) has suggested that the circadian variation in SWD activity is linked with sleep, although it has also been argued that while seizure activity coincides with activity of the master circadian clock, the suprachiasmatic nucleus (SCN), the sleep/wake cycle itself is not likely responsible for peaks in seizure activity (Quigg et al. 1998; Quigg et al. 2000). Furthermore, Sadleir et al. (2009) concluded that while absence seizures in humans are partially influenced by level of arousal, with increased SWD activity during drowsiness and sleep, these variables only partly explain the variation seen in circadian SWD activity. Although stress is the most frequently cited trigger for seizures, fatigue and sleep deprivation are also frequently cited as triggers, and Frucht et al. (2000) showed that stress, fatigue, and sleep deprivation were all positively correlated, suggesting that a similar mechanism of action underlies both stress and sleep deprivation as seizure generators. Interestingly, both *Scn8a*$^{med/+}$ mutants and *Scn1a*$^{RH/+}$ mutants show sleep deficits, particularly in REM sleep (Papale et al. 2010; Papale et al. 2013), which may contribute to spontaneous seizure activity in these mutants. However, future studies are needed to determine if a single acute stressor can change normal sleep patterns.

It is also possible that HPA axis activity is driving this long-lasting change in SWD activity in the *Scn8a*$^{med/+}$ mutants. HPA axis activity is also affected by the SCN and follows a circadian rhythm (Kalsbeek et al. 2011), and HPA axis activity in the *Scn8a*$^{med/+}$ mutants tracks with the SWD activity, as we observed a peak in baseline SWD activity from 5-7 pm which corresponds to the peak in CORT levels. Furthermore, the increase in the evening SWD activity following acute stress exposure in the morning is accompanied by increased 7 pm CORT levels in the *Scn8a*$^{med/+}$ mutants. There is evidence that seizure activity can increase HPA axis activity for up to a day. For

example, An et al. (2003) showed that following seizure activity in seizure-sensitive gerbils, CRH immunoreactivity does not return to pre-seizure levels until 24 hours later, and Wu et. al. (2012) demonstrated that pilocarpine-induced status epilepticus (SE) increased CRH gene expression significantly for up to a day after the SE. Even more intriguingly, a single 30-minute restraint stress can change CRH receptor 1 (CRHR1) expression for at least 24 hours, with reductions in ligand binding still seen 7 days later (Greetfeld et al. 2009). Post-stress evening levels of CORT were elevated in both the *Scn1a*$^{RH/+}$ mutants and the WT C57BL/6J littermates, suggesting a similar pattern as that shown in **Figure 5.1** where altered anxiety levels are driving the genotype-specific changes seen in the *Scn8a*$^{med/+}$ mutants, whereas the changes seen in the *Scn1a*^{RH} line are background-specific. Further investigation will be required to tease out the exact nature of the mechanistic pathways driving this extended response to an acute stressor. Interestingly, it has also been reported in humans that daily stress events and perceived stress levels are strongly associated with seizures over both the short-term and up to 24-hours later (Temkin and Davis 1984), highlighting the important therapeutic potential of elucidating these mechanisms of acute stress-seizure interaction over both the short-term and long-term.

5.3 VGSCs and Early Life Experience

Due to the time intensive nature of these experiments, we only examined the effect of early life experience on the *Scn8a*$^{med/+}$ mutants. Stressful events early in life can negatively affect seizure outcome in adulthood (Frye and Bayon 1999; Edwards et al. 2002; Huang et al. 2002; Lai et al. 2006; Salzberg et al. 2007; Jones et al. 2009), while

environmental enrichment has been shown to improve seizure outcome (Korbey et al. 2008; Korgan et al. 2013). Although we used the maternal separation (MS) paradigm, the mice in the MS groups received larger amounts of maternal care, an increasingly common observation in some mouse strains (Millstein and Holmes 2007; Own and Patel 2013). Increased maternal care, particularly grooming and licking of the pups, has been associated with increased stress resistance, lower levels of emotionality, and increased cognitive functioning in adults (Ladd et al. 2000; Sanchez et al. 2001; Champagne et al. 2008). While the MS paradigm in our study and the resulting increase in maternal care led to several improvements in adult outcome, we focus here on the interactions and influences of the *Scn8a* VGSC mutation.

While the MS paradigm resulted in increased seizure thresholds in *Scn8a*$^{med/+}$ mutants and WT littermates, the increase was much higher in the mutants, suggesting that the *Scn8a* mutation was acting synergistically with the early life experience to alter seizure thresholds. It is already known that *Scn8a*$^{med/+}$ mutants have higher seizure thresholds than their WT littermates due to decreased excitatory signaling (Makinson et al., under review), yet the MS *Scn8a*$^{med/+}$ mutants show a further increase in seizure thresholds as a result of their early life experience. Expression levels of *Scn8a* begin around P10 in the mouse (Gazina et al. 2010), a period that overlapped with our period of MS. While we have only just begun to understand the expression pattern of *Scn8a* during development, and more research is required to better understand the factors that influence this gene's expression, it is possible that the events and experiences during development could change that expression pattern. It has already been established that early life experiences can directly change excitability in certain brain regions, and excitability in

the brain is controlled by ion channels. In a cross-fostering study with the EL mouse, EL pups who had been cross-fostered and received higher levels of maternal care showed decreased activity levels in the hippocampus and cortex as adults (Leussis and Heinrichs 2009). EL mice exposed to environmental enrichment also demonstrated decreased reactivity of the hippocampus after exposure to tail suspension (Korbey et al. 2008). Maternal separation in Wistar rats resulted in enhanced burst firing of hippocampal pyramidal neurons (Ali et al. 2013), showing that adverse events can increase excitability. As our paradigm more closely modeled enrichment rather than adversity, we propose that the MS *Scn8a*$^{med/+}$ mutants had decreased neuronal excitability as a result of their early life experience, which increased their resistance to seizures in adulthood. The MS WT littermates also had slightly increased thresholds, but the *Scn8a*$^{med/+}$ mutants had the added advantage of decreased excitability due to the *Scn8a* mutation. Thus, an advantageous early life experience affected the *Scn8a*$^{med/+}$ mutants more strongly.

Another interesting result from the MS study was that the early life experience was able to unmask genotype differences not seen in our previous *Scn8a*$^{med/+}$ study (Sawyer *et al.* 2014). These differences were seen in both the DH and MS groups of *Scn8a*$^{med/+}$ mutants suggesting that the experience of daily handling and weighing was sufficient to elicit the appearance of a new phenotype. Based on the data presented in this thesis, the male *Scn8a*$^{med/+}$ mutants had a larger CORT response to a stressor and spent more time floating in the forced swim test as compared to WT littermates. In the literature, daily handling of rats has resulted in an anxiolytic effect in the open field test and elevated plus-maze (Schmitt and Hiemke 1998), and daily handling of rat pups has resulted in decreases in multiple depressive-like and anxiety-like behaviors (Boufleur et

al. 2013). In contrast, daily handling of C57BL/6J mice from P2 until P14 resulted in an increased susceptibility to experimental autoimmune encephalomyelitis (Columba-Cabezas et al. 2009). It is clear that daily handling permanently alters neural circuitry and resulting behavior, and in our study, the alterations due to the daily handling uncovered further phenotypes attributable to an *Scn8a* mutation. It is possible that in our model, the daily handling reduced anxiety levels which are normally increased in *Scn8a*$^{med/+}$ mutants. Indeed, the magnitude of differences in anxiety-like behaviors between the Scn8a$^{med/+}$ mutants and WT littermates were decreased in this study compared to our previous study. The decrease in these anxiety levels could have changed HPA axis function and the manifestation of depressive-like behavior that was observed in the daily handled *Scn8a*$^{med/+}$ mutants. In a future study, it could be investigated whether a reduction in anxiety levels in adult non-handled *Scn8a*$^{med/+}$ mutants would be sufficient to recapitulate these additional genotype effects.

Another interesting aspect of our early life study was the observed interaction between sex and genotype in the DH animals. Males and females performed differently in the novel cage paradigm, but the directionality of the differences depended on the genotype. WT females were less active and displayed decreased amounts of exploratory behaviors than the WT males, while *Scn8a*$^{med/+}$ females were more active and explored more than the mutant males. Normally, female rodents show less interest in novelty than males (Frick and Gresack 2003), which was recapitulated by the behavior of the WT females. Therefore, it appears that the *Scn8a* mutation is affecting the female response to novelty. Interestingly, the *Scn8a*$^{med/+}$ males spent less time exploring in the novel cage paradigm than the WT littermates, consistent with other anxiety-like behaviors

attributable to the *Scn8a* mutation in males. So not only is *Scn8a* affecting female behavior, but it is affecting behavior in a sex-specific manner. Hu et al. (2012) recently showed that $Na_v1.6$ expression is reduced and $Na_v1.1$ expression is increased in dorsal root ganglion neurons of a estrogen receptor knockout mouse, suggesting that the presence or absence of estrogen can directly affect VGSC expression. In the CNS, the effects of estrogen on learning and memory via hippocampal circuits have been studied in detail (Roepke et al. 2011), but to our knowledge, no one has examined the effect of estrogen on VGSC expression in the brain. As estrogen is generally thought of as a neurosteroid that can increase excitability (Herzog 2007), perhaps the estrogen in the *Scn8a*$^{med/+}$ females is compensating for the decreased excitability due to the VGSC mutation, explaining why the *Scn8a*$^{med/+}$ females are more exploratory than the *Scn8a*$^{med/+}$ males. Furthermore, the increased excitability due to estrogen combined with the decreased excitability due to the *Scn8a* mutation may be interacting in ways that make the *Scn8a*$^{med/+}$ females different from the WT females. An interesting line of future studies could pursue the effect of female sex hormones on $Na_v1.6$ expression within the female brain.

5.4 VGSCs and Behavior

While behavior was not originally a major focus of this dissertation, several interesting behavioral results led us to further investigate the role of VGSCs in behavior. Furthermore, none of the animals in the behavioral studies described herein were acclimated prior to behavioral testing, so the behavioral tests include a mildly stressful component and can be thought of as manifestations of behavior in mildly stressful

situations. While both the *Scn8a*$^{med/+}$ mutants and *Scn1a*$^{RH/+}$ mutants demonstrate behavioral abnormalities, the resulting behavioral profiles are very different as discussed in more detail below.

5.4.1 Anxiety- and Depressive-Like Behaviors

The *Scn8a*$^{med/+}$ mutants show higher levels of anxiety-like behaviors, while the *Scn1a*$^{RH/+}$ mutants showed no obvious measures of increased anxiety. Interestingly, high levels of anxiety in other animal models of epilepsy appears to be highly reproducible (Heinrichs 2010). For example, the absence seizure susceptible WAG/Rij rats show increased agitation, decreased exploration, increasing grooming, and hyperlocomotion in response to novelty stress (Midzyanovskaya et al. 2005), similar to behaviors observed in the *Scn8a*$^{med/+}$ mutants. These findings complement the human literature in which anxiety disorders are often co-morbid with epilepsy (Goldstein and Harden 2000; Vazquez and Devinsky 2003; Kanner 2009). While we did not find increased anxiety in the *Scn1a*$^{RH/+}$ mutant, alterations in anxiety have been described in other *Scn1a* mutants (Han et al. 2012; Ito et al. 2012), suggesting that the milder *Scn1a*$^{RH/+}$ mutation is not severe enough to affect anxiety-like behaviors. As described previously, it is possible that the higher anxiety level seen in the *Scn8a*$^{med/+}$ mutants is driving the worsening of the seizure outcome in response to acute stress. The anxiety response and stress response share many common mediators, including the HPA axis and activation of limbic regions, suggesting areas that could be explored in future mechanistic studies examining the relationship between anxiety and the stress-induced seizure response.

In addition to anxiety, depression is often highly comorbid with epilepsy in human patients (Kanner 2005) as well as in animal models (Epps and Weinshenker 2013). However, we found no evidence of depressive-like behaviors in either the male *Scn8a*$^{med/+}$ mutants or the *Scn1a*$^{RH/+}$ mutants in behavioral tests under normal laboratory conditions. Following daily handling during the postnatal period, however, a genotype difference did emerge in the male *Scn8a*$^{med/+}$ mutants which suggested greater levels of depressive-like behaviors due to an *Scn8a* mutation depending on early life experience. Like anxiety, the HPA axis and activity in limbic areas could be linking factors between epilepsy and depression. The HPA axis is usually hyperactive in cases of major depression (De Kloet 2004; Swaab et al. 2005), while anxiety disorders may involve no changes in baseline HPA axis activity (Vreeburg et al. 2010; Staufenbiel et al. 2012). These findings in human studies corroborate the findings described within this dissertation. There were no changes in baseline HPA axis activity in the more anxious *Scn8a*$^{med/+}$ mutants, while following an altered early life experience, male *Scn8a*$^{med/+}$ mutants demonstrated depressive-like behaviors as well as a hyperactive HPA axis response to an acute stress. Overall, our results indicate that *Scn8a* may be playing a role in both anxiety- and depressive-like behaviors, depending on early life factors, while a mild *Scn1a* mutation appears to affect neither type of behavior.

5.4.2 Social Behavior

Social behavior was only analyzed in the *Scn1a*$^{RH/+}$ mutants. Emerging evidence has linked *SCN1A* mutations with behaviors seen in autism-spectrum disorders (ASDs)

(Weiss et al. 2003; O'Roak et al. 2012), and autistic-like behavior has been recently described in an *Scn1a* knockout mouse and a mouse with a nonsense mutation in *Scn1a*, both mouse models of a severe form of epilepsy, Dravet Syndrome (DS) (Han et al. 2012; Ito et al. 2012). Furthermore, recent evidence has more broadly linked ASDs with deficits in inhibitory signaling (Chao et al. 2010; Paluszkiewicz et al. 2011). Han et al. (2012) have presented evidence that the social deficits in the mouse model of DS are caused by a lack of inhibitory signaling in forebrain interneurons, and that increasing GABAergic signaling, such as with a low-dose benzodiazepine treatment, can attenuate these social deficits. Our behavioral studies with the *Scn1a*$^{RH/+}$ mutants demonstrate hyperactivity and mild social deficits in this mouse model of GEFS+. Autistic-like behaviors reported in human patients with GEFS+ are less common (Dixon-Salazar et al. 2004; Tan et al. 2012), but our results suggest that these traits may exist in a less severe form in GEFS+ and may therefore be easily overlooked in the clinical setting. Our study provides the first evidence of social deficits in a GEFS+ model.

5.4.3 Other Behavior Findings

In additional to social deficits, *SCN1A* mutations have also been linked with cognitive deficits (Harkin et al. 2007), and cognitive deficits have been reported in mouse models of DS (Han et al. 2012; Ito et al. 2012). We found mild impairments in learning and memory in our GEFS+ model, *Scn1a*$^{RH/+}$ mutants. The $Na_v1.1$ VGSC is important for inhibitory signaling in interneurons, particularly parvalbumin (PV) interneurons (Dutton et al. 2012). Studies showing selective impairment of PV interneurons in the

hippocampus can result in learning and memory impairments (Korotkova et al. 2010; Murray et al. 2011), providing a possible mechanistic explanation for the results presented herein. Our study again provides the first evidence of cognitive deficits in a GEFS+ model.

Interestingly, we also found novel behavioral phenotypes in the *Scn1a*$^{RH/+}$ mutants. The *Scn1a*$^{RH/+}$ mutants showed deficits in prepulse inhibition (PPI) of the acoustic startle response, a measure of sensorimotor gating. While PPI deficits have been strongly linked with schizophrenia, studies of PPI in other disorders, such as autism, are scarce (Kohl et al. 2013). Hyperactivity, deficits in PPI, and deficits in cued fear conditioning have all been linked to alterations in PV interneuron signaling (Shamir et al. 2012), supporting the manifestation of these phenotypes in the *Scn1a*$^{RH/+}$ mutant. Also, a study by Ma et al. (2010) showed that kindling of the prefrontal cortex resulted in PPI deficit and hyperactivity, two phenotypes present in the *Scn1a*$^{RH/+}$ mutants. As *Scn1a* expression is high in PV interneurons in the prefrontal cortex (Dutton et al. 2012), activity in this area may be responsible for both of these phenotypes in the *Scn1a*$^{RH/+}$ mice. Further studies could follow-up the results presented in this dissertation by using *Scn1a* conditional knockout mice to localize specific brain regions responsible for the PPI deficits and hyperactivity seen in the *Scn1a*$^{RH/+}$ mutants.

Another novel finding from our studies was the risk-taking behavior demonstrated by the *Scn1a*$^{RH/+}$ mutants. We found that the *Scn1a*$^{RH/+}$ mutants froze less in response to a predator odor exposure and seemingly ignored the danger of a virtual cliff. Personal observations during the novel object recognition test also suggested that the *Scn1a*$^{RH/+}$ mutants tend to advance into unfamiliar situations without first stopping to assess risk.

Risk-taking behavior in epilepsy has been poorly studied. One recent study by Alfstad et al. (2011) did show an increase in risk-taking behavior in young boys with epilepsy. Studies of risk-taking behavior on its own has led to the implication of the prefrontal cortex in this type of behavior (Studer et al. 2013), further suggesting that abnormal PV interneuron signaling in the prefrontal cortex may be driving more than one phenotype seen in the *Scn1a*$^{RH/+}$ mutants. In fact, risk-taking behavior may be closely associated with hyperactivity, as ADHD patients show high levels of impulsivity (Ibanez et al. 2012). This intriguing new phenotype in the *Scn1a*$^{RH/+}$ mutants opens up new avenues of investigation into comorbidities associated with *Scn1a* dysfunction.

5.5 Future Directions

The results from the studies described herein have resulted in numerous new questions and opportunities for future directions. While the possibilities for future research are numerous, I have selected four possible avenues for follow-up studies. This list is by no means exhaustive, but includes some of the questions that I found very interesting.

5.5.1 Further Characterization of the HPA Axis of VGSC Mutants

In the current studies, HPA axis activity was assessed primarily through examination of CORT levels. While CORT levels can certainly be used as a marker of HPA axis activity, they can only provide a small window into a complex system

containing components interacting on multiple levels. For example, although basal levels of CORT in both the *Scn8a*$^{med/+}$ and *Scn1a*$^{RH/+}$ mutants were normal, we do not know if the CORT receptors, the MRs and GRs, are expressed normally in the brains of these mutants. Dysregulation in the balance of activity between these two receptor subtypes has been implicated in numerous disorders (De Kloet et al. 1998). So while plasma CORT levels in the VGSC mutants may be normal, the effects of the CORT on brain processes through action at GRs and MRs would have to be further examined. As MR-mediated events appear to be pro-convulsive and GR-mediated events appear to return excitation levels to normal (Joels 2009), an imbalance in these receptors could definitely affect the seizure response to a stressor. Future experiments could use genetic or immunohistochemical techniques to analyze the balance of MR and GR receptors in the brains of VGSC mutants, paying special attention to limbic areas such as the hippocampus and amygdala. In addition, recent evidence has also implicated a GR chaperone protein, FKBP5, in certain disorders involving HPA axis dysregulation, particularly post-traumatic stress disorder (Gillespie et al. 2009). While many other components of the HPA axis have been investigated in epilepsy models, no one has looked at the role of FKBP5 in epilepsy and/or the seizure response to stress to date.

The role of CRH in the seizure response to stress in VGSC mutants is also worthy of future study. CRH is primarily pro-convulsive, particularly in limbic regions (Maguire and Salpekar 2013). Analysis of CRH release and expression levels of the two CRH receptors, CRHR1 and CRHR2 could also provide further insight into the seizure response to a stressor. CRH is also highly implicated in anxiety disorders (Fox and Lowry 2013), and may particularly be driving the seizure response to stress in the

Scn8a$^{med/+}$ mutants in conjunction with the increased levels of anxiety seen in this model. Again, genetic and/or immunohistochemical techniques could be applied to further elucidate the role of the CRH system in the connection between stress and epilepsy.

5.5.2 Role of Anxiety in the Stress Response of VGSC Mutants

The role of anxiety in worsening the seizure response to stress in the *Scn8a*$^{med/+}$ mice is intriguing. An interesting follow-up study could try to ameliorate the seizure response to stress by targeting pathways involved in the anxiety response. For example, one such follow-up experiment could involve administration of an anxiolytic drug prior to restraint stress and a seizure induction paradigm to see if ameliorating the anxiety response to the stressor could ameliorate the resulting seizure activity. While anxiety is typically thought to be co-morbid with epilepsy and focus has been on how high anxiety levels may impact quality of life in epilepsy patients, there has been little focus on anxiety actually being the driving force behind stress-induced seizures. For example, in a paper by Beyenburg et al. (2005), clinicians are encouraged to differentiate between ictal, postictal and interictal anxiety, but no mention is made of the possibility that anxiety could be driving seizure activity as a type of "preictal" anxiety. While epilepsy is frequently thought of as being a causal factor for anxiety, the converse relationship has not been well explored. In addition to the type of experiment mentioned above, it would be interesting to evaluate seizure susceptibility in animal models of anxiety. Furthermore, as mentioned in the previous section there is also a close relationship between anxiety and HPA axis function, particularly involving CRH. A better characterization of HPA

axis activity in the VGSC mutants could therefore help us tease apart the pathways responsible for the stress response to seizures observed in these experiments.

The daily handling of *Scn8a*$^{med/+}$ mutants also resulted in novel phenotype differences in adult animals. Adult male *Scn8a*$^{med/+}$ mutants that had been handled daily as pups had a higher CORT response to a stressor and floated more in the forced swim test. As daily handling can decrease anxiety levels in rodents (Schmitt and Hiemke 1998; Boufleur et al. 2013) and daily handled *Scn8a*$^{med/+}$ mutants did show reduced anxiety levels as compared to non-handled *Scn8a*$^{med/+}$ mutants, it is possible that a reduction in anxiety levels allowed the unmasking of a new phenotype that was otherwise overshadowed by the anxiety phenotype. Therefore, it would also be interesting to use anxiolytic treatments in adult *Scn8a*$^{med/+}$ mutants to see if the genotype differences in the stress CORT response and floating behavior in the forced swim test could be unmasked in adulthood independently of early life experience.

5.5.3 Effect of Stress on Sleep Patterns in VGSC Mutants

In addition to stress, sleep deprivation is a commonly cited trigger for seizures, and the mechanisms by which stress and sleep disturbances work to trigger seizures may be connected (Frucht et al. 2000). Given the observed circadian rhythm of SWDs in the *Scn8a*$^{med/+}$ mutants, we have wondered what role sleep is playing in the stress response. After a morning stressor, the evening peak of spontaneous SWD activity was broadened in the *Scn8a*$^{med/+}$ mutants. While this increase in circadian SWD activity was correlated with elevated 7 PM levels of CORT, it is also possible that the acute restraint stress in the

morning affected sleep patterns in these mice. All stressors in these experiments were conducted between 8 and 11 am to coincide with a period of low CORT levels in rodents. However, this is also during the light period of rodents, a period correlated with increased levels of sleep and reduced activity. Stressing the animals during a period when they would normally be sleeping could have resulted in mild sleep deprivation. Therefore, characterization of the sleep patterns following an acute restraint stress would allow us to better understand the effects of VGSC on the stress response. We have already been shown that both *Scn8a* and *Scn1a* mutants have alterations in their baseline sleep architecture (Papale et al. 2010; Papale et al. 2013), and the response of mild sleep deprivation has been examined in these mutants as well. However, there has been no characterization of the sleep response to an acute restraint stress in neither our models nor other models of epilepsy. Given the fact that it has been proposed that stress, sleep deprivation, and fatigue work through common mechanisms to induce seizure activity (Frucht et al. 2000), it would be an interesting line of investigation to pursue further.

5.5.4 Role of Sex Hormones in VGSC Expression and Function

While not related directly to the interaction between stress and epilepsy, the effects of sex hormones on VGSC expression and function would be an interesting line of research to pursue. The sex effects in our early life stress study raise some interesting questions into how sex hormones are directly affecting excitability within the brain and specifically how they are affecting VGSC expression and function. Evidence that the absence of an estrogen receptor can alter both $Na_v1.6$ and $Na_v1.1$ expression in the

periphery (Hu et al. 2012) suggests that estrogen could be having direct effects on VGSC expression and even function. Other studies of gonadotropin-releasing hormone (GnRH) neurons in the hypothalamus reveal that estrogen can directly attenuate sodium currents and affect neuron firing rates (Wang and Kuehl-Kovarik 2012). While it has been reported the idiopathic epilepsy is more common in women (Christensen et al. 2005), only one study has reported sex differences in *SCN1A*-derived epilepsy (Ohmori et al. 2003). In the studies described herein, sex differences were only examined in the *Scn8a*^{med} line. Future studies could examine sex differences in the *Scn1a*^{RH} line and the role of estrogen in modulating VGSC expression in both VGSC mutants.

5.6 Overall Conclusions

The studies described herein show that a genetic predisposition to epilepsy, and mutations in voltage-gated sodium channels (VGSCs) in particular, can affect the stress response, the seizure outcome following a stressor or altered early life experience, and behavior. As shown in **Figure 5.3**, multiple factors are involved in the seizure response to stress. Stress (red arrow) can affect the brain in ways that results in altered output in the form of HPA axis activity and behavior. These responses can be either adaptive or maladaptive, depending on an individual's genetic makeup and early life influences. Furthermore, this complex interplay of multiple factors and responses can affect the seizure response to a stressor (orange arrow), resulting in either an improved seizure outcome or a worsened seizure outcome. It is clear from the experiments described in this dissertation that the dysfunction of specific VGSCs does play a role in this chain of events by altering the balance between excitation and inhibition within the brain's neural

circuitry. Thus, having a genetic predisposition to epilepsy affects multiple systems at multiple levels, altering the stress response and the seizure response to stress. This research highlights the importance of using genetic models of epilepsy to investigate the relationship between stress and epilepsy rather than depending on wildtype models with normal levels of excitability prior to stress inducement.

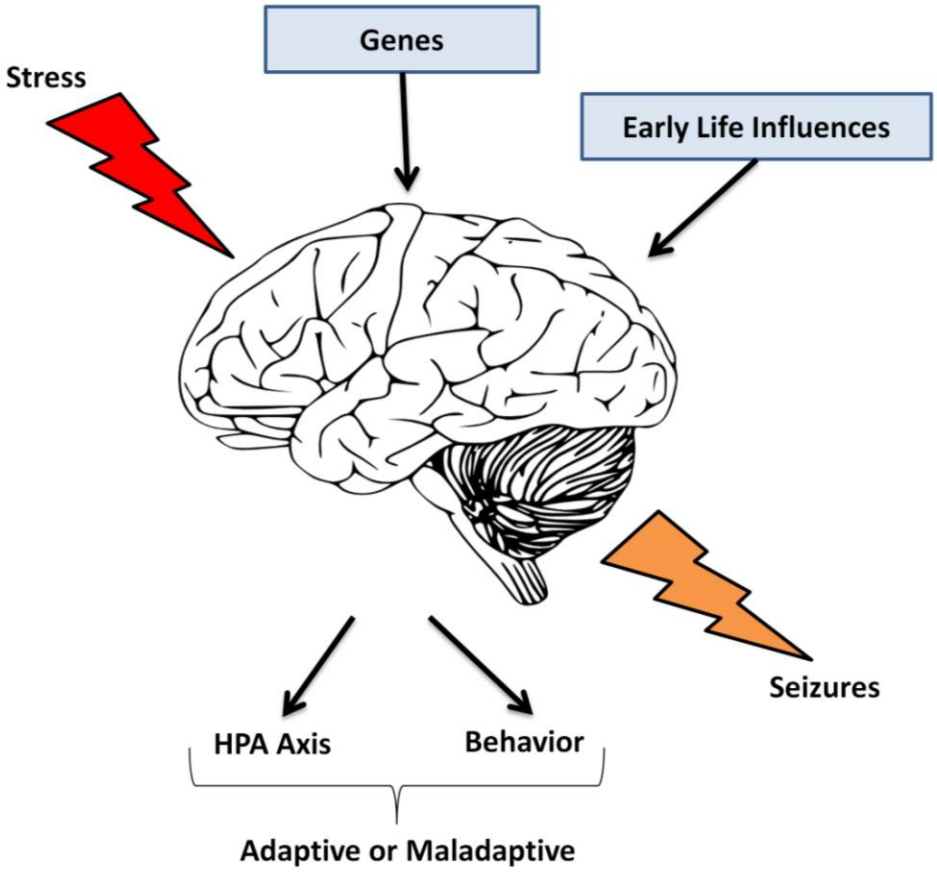

Figure 5.3 Responses to Stress. The resilience or vulnerability of any one individual to stress in adulthood can depend upon that individual's genetic makeup and early life experiences.

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APPENDIX A: FULL DATA SET FROM *SCN8A*^{MED} MS EXPERIMENTS

A1 Baseline Spontaneous SWD Activity from MS Experiments

Table A1.1 Baseline EEG Measures

	DH WT	DH *Scn8a*$^{med/+}$	MS WT	MS *Scn8a*$^{med/+}$
MALES:				
Total Number of SWDs in a 24-Hr Period	21.8 (±7.8)	*263.6 (±68.6)	38.2 (±17.9)	*369.4 (±83.9)
Average Duration of SWDs (s)	2.0 (±0.1)	*3.1 (±0.2)	1.9 (±0.2)	*3.3 (±0.3)
Total Time Seizing in a 24-Hr Period (s)	47.8 (±19.9)	*898.6 (±242.6)	83.8 (±40.7)	*1363.0 (±368.2)
FEMALES:				
Total Number of SWDs in a 24-Hr Period	20.3 (±7.1)	*360.7 (±51.1)	14.6 (±5.7)	*363.2 (±55.5)
Average Duration of SWDs (s)	2.1 (±0.1)	*3.0 (±0.2)	1.9 (±0.1)	*3.1 (±0.2)
Total Time Seizing in a 24-Hr Period (s)	42.5 (±17.1)	*1090.2 (±177.4)	28.0 (±11.1)	*1202.3 (±248.8)

*p<0.05 Significant Genotype difference (WT vs. *Scn8a*$^{med/+}$ within condition and sex)
- There was no observed effect of MS
- There was no observed effect of sex

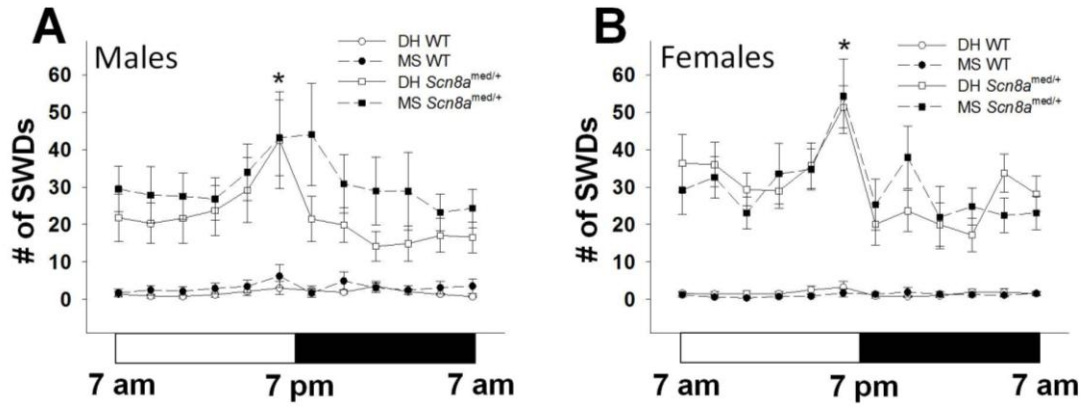

Figure A1.1 MS does not affect circadian rhythm of SWDs in male or female *Scn8a*$^{med/+}$ mutants. The baseline 24-hour circadian rhythm of absence seizures in *Scn8a*$^{med/+}$ mutants is not affected by MS in either (A) males or (B) females. There are also no significant sex differences between the two groups. In both groups of *Scn8a*$^{med/+}$ mutants, there is a significant peak of activity from 5-7 pm. n=9 all groups; *p<0.05 vs. all other time points (except for 3-5 pm in males); Error bars represent SEM.

A2 Post-Stress Response in MS Experiments

Table A2.1 Post-Stress Measures

	DH WT	*DH *Scn8a*$^{med/+}$	MS WT	*MS *Scn8a*$^{med/+}$
MALES:				
Number of SWDs while in Restraint Tube	0	*1.3 (±0.6)	0	*1.4 (±0.5)
Struggle Time in the Restraint Tubes (s)	28.1 (±2.5)	*19.6 (±4.3)	28.0 (±3.8)	*18.3 (±2.2)
Latency to first SWD following onset of Stress (s)	218.9 (±82.5)	*59.1 (±29.2)	177.2 (±72.4)	*31.9 (±11.4)
FEMALES:				
Number of SWDs while in Restraint Tube	0	*3.4 (±1.0)	0	*2.8 (±0.7)
Struggle Time in the Restraint Tubes (s)	32.4 (±3.6)	*27.3 (±2.9)	32.5 (±3.5)	*21.0 (±3.2)
Latency to first SWD following onset of Stress (s)	146.5 (±74.1)	*17.0 (±4.3)	141.9 (±54.4)	*16.2 (±2.6)

*$p<0.05$ Significant Genotype difference (WT vs. *Scn8a*$^{med/+}$ within condition and sex)

- There was no observed effect of MS
- There was no observed effect of sex

n=9 all groups

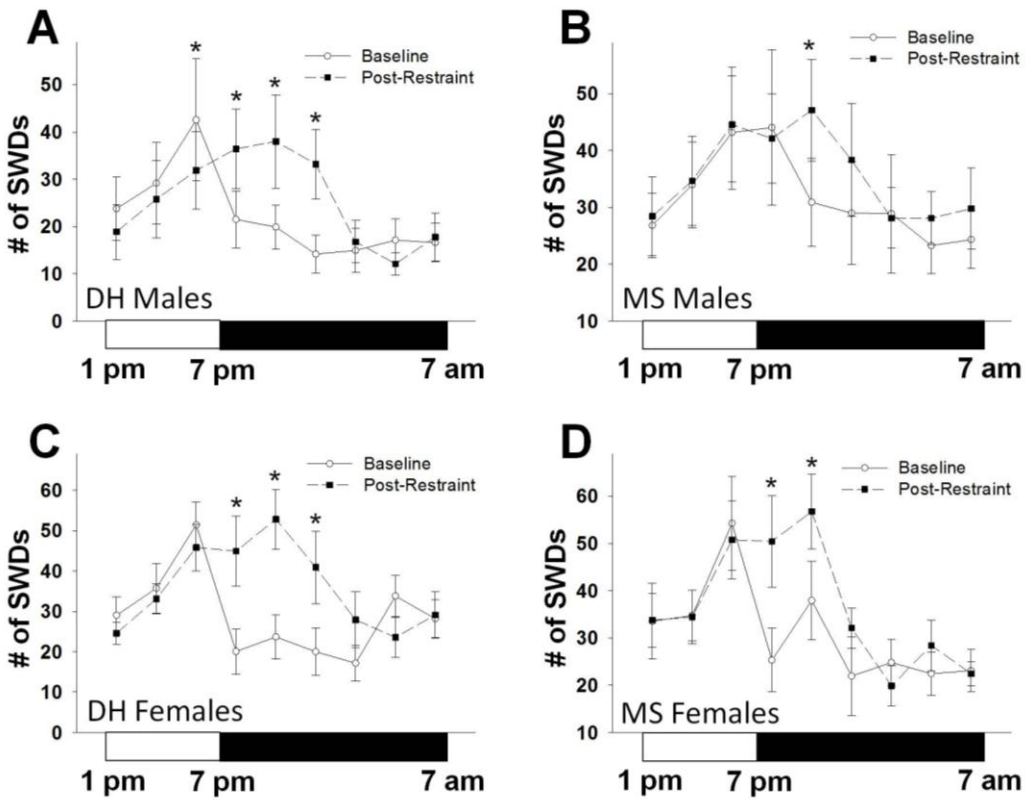

Figure A2.1 MS does not affect the stress-induced long-lasting effects to the circadian rhythm of SWDs in male or female *Scn8a*$^{med/+}$ mutants. Following a 20-minute acute restraint stress in the morning and a return to baseline levels, all groups of *Scn8a*$^{med/+}$ mutants show a broader peak of SWD activity during the evening rhythm of SWD activity. There were no main effects of MS nor main effects of sex. All groups did show a main effect of restraint stress. (A) DH males; (B) MS males; (C) DH females; (D) MS females. n=9 all groups; *p<0.05 vs. baseline. Error bars represent SEM.

A3 Flurothyl Results from MS Experiments

Table A3.1 Flurothyl Results

	DH WT	DH *Scn8a*$^{med/+}$	MS WT	MS *Scn8a*$^{med/+}$
MALES:				
Latency to Myoclonic Jerk (min)	1.8 (±0.2)	*2.4 (±0.1)	1.9 (±0.2)	*2.5 (±0.1)
Latency to first Generalized Seizure (min)	4.2 (±0.2)	*7.9 (±0.5)	4.0 (±0.2)	*7.7 (±0.5)
Latency to GTCS with Bouncing and HLE (min)	11.3 (±0.1)	*12.4 (±0.3)	10.9 (±0.3)	11.6 (±0.6)
Number of Seizures Prior to GTCS w/ HLE	1.9 (±0.1)	1.2 (±0.2)	2.2 (±0.4)	*1.1 (±0.3)
FEMALES:				
Latency to Myoclonic Jerk (min)	2.0 (±0.2)	*2.6 (±0.1)	2.3 (±0.2)	2.5 (±0.1)
Latency to first Generalized Seizure (min)	4.4 (±0.2)	*#6.6 (±0.3)	4.6 (±0.2)	*6.8 (±0.2)
Latency to GTCS with Bouncing and HLE (min)	11.3 (±0.3)	11.5 (±0.6)	12.0 (±0.2)	*+13.1 (±0.2)
Number of Seizures Prior to GTCS w/ HLE	2.3 (±0.3)	*1.4 (±0.3)	2.9 (±0.3)	2.0 (±0.3)

*p<0.05 Significant Genotype difference (WT vs. *Scn8a*$^{med/+}$ within condition and sex)

#p<0.05 Significant Sex difference (Male vs. Female within DH)

+p<0.05 Significant MS effect (DH vs. MS within same genotype and sex)

n=12 all groups

A4 Picrotoxin Results from MS Experiments

Table A4.1 Picrotoxin Results

	DH WT	DH *Scn8a*$^{med/+}$	MS WT	MS *Scn8a*$^{med/+}$
MALES:				
Latency to Stage 1 Seizure (min)	8.0 (±0.6)	*10.2 (±0.9)	7.1 (±0.5)	*19.0 (±5.6)
Latency to Stage 2 Seizure (min)	12.5 (±0.7)	14.5 (±0.4)	13.0 (±0.7)	**25.4 (±6.0)
Latency to Stage 3 Seizure (min)	12.7 (±0.7)	15.4 (±0.5)	13.0 (±0.7)	**29.8 (±5.8)
Latency to Stage 4 Seizure (min)	12.7 (±0.7)	18.8 (±2.1)	13.0 (±0.7)	**30.5 (±5.7)
Latency Stage 5 Seizure (min)	15.1 (±3.0)	*43.7 (±7.0)	18.0 (±4.7)	*50.2 (±5.2)
Racine Score	4.6 (±0.2)	*3.7 (±0.1)	4.2 (±0.2)	*3.0 (±0.4)
FEMALES:				
Latency to Stage 1 Seizure (min)	7.3 (±0.6)	*10.5 (±0.9)	8.2 (±0.7)	9.8 (±0.9)
Latency to Stage 2 Seizure (min)	14.7 (±1.2)	*#22.8 (±4.7)	15.4 (±1.2)	17.5 (±1.3)
Latency to Stage 3 Seizure (min)	16.0 (±1.3)	*#24.2 (±4.5)	16.0 (±1.3)	19.8 (±1.3)
Latency to Stage 4 Seizure (min)	16.2 (±1.3)	*#40.0 (±6.4)	20.8 (±5.1)	*34.8 (±5.0)
Latency Stage 5 Seizure (min)	#48.3 (±7.7)	#60.0 (±0.0)	56.7 (±3.3)	56.7 (±3.3)
Racine Score	4.3 (±0.2)	*3.4 (±0.3)	4.1 (±0.1)	3.9 (±0.1)

*p<0.05 Significant Genotype difference (WT vs. *Scn8a*$^{med/+}$ within condition and sex)

#p<0.05 Significant Sex difference (Male vs. Female within DH)

+p<0.05 Significant MS effect (DH vs. MS within same genotype and sex)

n=12 all groups

A5 Sex Differences in CORT Levels

Table A5.1 Sex Comparison of CORT Levels (all DH animals)

	Male WT	Male *Scn8a*$^{med/+}$	Female WT	Female *Scn8a*$^{med/+}$
Basal CORT (ng/mL)	13.3 (±0.8)	15.3 (±2.7)	*23.1 (±4.1)	*26.5 (±5.1)
CORT Response to Acute Restraint Stress (ng/mL)	426.6 (±22.3)	473.0 (±23.8)	*766.2 (±30.0)	*767.2 (±31.9)
Basal 7 PM CORT Levels (ng/mL)	130.8 (±12.3)	127.2 (±15.8)	*199.4 (±24.2)	*229.9 (±30.6)
Post-Stress 7 PM CORT Levels (ng/mL)	147.8 (±28.9)	154.1 (±33.9)	222.3 (±63.4)	160.3 (±64.2)

*$p<0.05$ Significant Sex difference (Male vs. Female within DH)

n=12 all groups

APPENDIX B: ADDITONAL DATA FROM *SCN1A*$^{RH/+}$ EXPERIMENTS

B1 Hyperactivity in other Behavioral Tasks in *Scn1a* R1648H

After demonstrating hyperactivity in the open field test, we have replicated the hyperactive phenotype of the *Scn1a*$^{RH/+}$ mutants in a number of other behavioral tasks (**Table B1.1**).

Table B.1 Hyperactivity in *Scn1a*$^{RH/+}$ Mutants in Other Behavioral Tasks

	Wildtype	*Scn1a*$^{RH/+}$	t value	p value
Open Field Portion of Novel Object Recognition				
Total Distance Travelled (m)	46.3 (±3.4)	60.9 (±2.7)	$t_{(22)}$=-3.390	p=0.003
Average Speed (cm/s)	7.7 (±0.6)	10.1 (±0.4)	$t_{(22)}$=-3.371	p=0.003
Time Spent Immobile (s)	150.3 (±17.0)	82.8 (±6.4)	$t_{(22)}$=3.725	p=0.001
Virtual Cliff Test				
Total Distance Travelled (m)	40.0 (±2.6)	52.3 (±2.8)	$t_{(22)}$=-3.232	p=0.004
Average Speed (cm/s)	6.7 (±0.4)	8.7 (±0.5)	$t_{(22)}$=-3.204	p=0.004
Time Spent Immobile (s)	218.6 (±18.5)	136.3 (±12.4)	$t_{(22)}$=3.689	p=0.001
Social Interaction				
Total Distance Travelled (m)	95.3 (±4.7)	120.1 (±8.5)	$t_{(22)}$=-2.547	p=0.018
Average Speed (cm/s)	5.3 (±0.3)	6.7 (±0.5)	$t_{(22)}$=-2.545	p=0.018
Time Spent Immobile (s)	464.9 (±34.3)	308.3 (±28.2)	$t_{(22)}$=3.524	p=0.002
Novel Cage				
Number of Immobile Episodes	55.3 (±4.4)	42.0 (±3.9)	$t_{(21)}$=2.263	p=0.034
Forced Swim Test				
Time Spent Struggling (s)	16.0 (±5.2)	35.5 (±8.4)	$t_{(21)}$=-1.920	p=0.069

B2 Additional Risk Assessment Tasks – *Scn1a* R1648H

B2.1 Methods

Novelty Suppressed Feeding Test

Twenty-four hours before the test, mice (12 *Scn1a*$^{RH/+}$ mutants and 12 WT littermates) were deprived of all food in their home cage. Prior to the test, a standard chow food pellet was weighed and then placed in the center of the open field apparatus. The test began immediately after each mouse was placed in the corner of the apparatus and latency to feed on the pellet was measured. The cutoff time of this test was set to 5 minutes. After 5 minutes, each mouse was returned to the home cage and the amount of food eaten was measured by weighing the food pellet a second time.

Modified Novelty Suppressed Feeding Test with Sucrose

We repeated the novelty suppressed feeding test with some modifications. Instead of food deprivation prior to the test, a reward item (sucrose-enhanced food pellet) was used. The experimental mice had previously been exposed to the sucrose-enhanced food pellets (see **Appendix B3**) and had shown preference for eating these pellets over standard mouse chow. Pellets were pre-weighed and placed into the center of the open field arena. Mice (12 *Scn1a*$^{RH/+}$ mutants and 12 WT littermates) were placed into a corner of the apparatus and latency to feed on the pellet was measured. The cutoff time of this test was set to 5 minutes. After 5 minutes, each mouse was returned to the home cage and the amount of food eaten was measured by weighing the food pellet a second time.

B2.2 Results

Within the 5-minute observation period of the test, none of the animals ate either the normal food (after a 24-hour fast) nor the sucrose-enhanced food (no fast). Therefore, we were unable to assess the latency to eat the food in either task. The test would need to be repeated with a test time longer than 5 minutes. The results for the other measures scored are listed in Table B1. The food zone was designated as a 5-cm region around the food pellet. The center zone was the same dimensions as in the open field task. The 24-hour overnight fast did have an effect on the behavior of the *Scn1a*$^{RH/+}$ mutants, as they seemed to spend less time in the center of the open field and investigating the food (**Table B2.1**). These differences between the genotypes were not seen in any of the open field tasks that did not involve fasting, particularly the "Time in Center Zone" measure. As an example, a comparison of this task following a fast versus the modified version using a sucrose-enhanced pellet and no fasting shows very different results (Table B1). It's possible that the low glucose levels from the fast led to ketosis in these mice and this affected their behavior in this task. Our lab has previously shown that the ketone diet can affect *Scn1a*$^{RH/+}$ mutants (Dutton et al. 2011).

Table B2.1 Results of Risk Assessment Behavioral Tasks

	Wildtype	*Scn1a*$^{RH/+}$	t value	p value
Novelty-Suppressed Feeding Test 24-Hour Food Deprivation; Normal Food				
Total Time in Food Zone (s)	20.0 (±4.9)	11.7 (±1.1)	$t_{(22)}$=1.958	p=0.063
Latency to Enter Food Zone (s)	38.4 (±9.8)	44.2 (±14.8)	$t_{(21)}$=-0.336	p=0.740
Longest Visit to Food Zone (s)	4.3 (±0.7)	2.7 (±0.2)	$t_{(22)}$=2.205	p=0.038
Total Time in Center Zone (s)	36.5 (±5.3)	24.7 (±2.1)	$t_{(22)}$=2.062	p=0.051
Modified Novelty-Suppressed Feeding Test No Food Deprivation; Sucrose-Enhanced Food				
Total Time in Food Zone (s)	10.4 (±1.2)	8.2 (±0.9)	$t_{(22)}$=1.492	p=0.150
Latency to Enter Food Zone (s)	23.4 (±6.8)	24.1 (±4.7)	$t_{(22)}$=-0.084	p=0.934
Longest Visit to Food Zone (s)	1.7 (±0.1)	1.7 (±0.1)	$t_{(21)}$=-0.073	p=0.942
Total Time in Center Zone (s)	33.3 (±3.3)	26.3 (±2.7)	$t_{(22)}$=1.632	p=0.117

B3 Depressive-Like Behavioral Tasks – *Scn1a* R1648H

B3.1 Methods

Highly Palatable Food Preference Paradigm

During three consecutive days, a highly palatable food (HPF) preference test was performed by offering a choice between a pre-weighed (~2 g) standard chow food (SCF) pellet and two 1-gram HPF pellets. The SCF pellet consisted of the normal rodent diet (3.3 kcal/g, 72.5% carbohydrates, 8.2% fat, 19.3% protein), while the HPF pellets were sucrose-enhanced (5-TUL, 3.44 kcal/g, 66.7% carbohydrate, 12.7% fat, 20.6% protein; Test Diet, PMI Nutrition International, St. Louis, MO, USA). The SCF and HPF pellets were placed in each mouse's home cage at 4 pm each day. The regular food was removed at the same time. One hour later, the SCF and HPF pellets were removed and replaced with normal food. The weights of the pellets were again measured to calculate the intake of both diets. Care was taken to look for spillage to measure as accurately as possible food intake.

B3.2 Results

In the forced swim test, there were no significant differences between *Scn1a*$^{RH/+}$ mutants and WT littermates in any of the measured parameters (**Fig. B3.1**A). Although there was a trend for the *Scn1a*$^{RH/+}$ mutants to struggle more than the WT littermates ($t_{(21)}$=-1.920, p=0.069), we believe that this is more likely due to the hyperactivity of the

Scn1a$^{RH/+}$ mutants (**Appendix B1**). The differences in the total time spent floating ($t_{(22)}$=0.507, p=0.617) and the latency to float ($t_{(22)}$=-1.221, p=0.235) were not difference between genotypes.

Due to the possibly of the hyperactivity affecting the outcome of the forced swim test, we decided to also test the *Scn1a*$^{RH/+}$ mutants in a highly palatable food (HPF) preference paradigm. Similar in concept to the sucrose preference test, mice are given a choice between regular rodent chow and a sucrose-enhanced HPF. There were no differences in the percentage of food eaten that was HPF (**Fig. B3.1**B). There was no main effect of genotype, nor was there a genotype-day interaction, but there was a main effect of day ($F_{(2,44)}$=25.445, p<0.001), indicating that all mice were increasing the percentage of HPF food they were eating across the three day testing period. This result was likely due to reduced eating on the first day due to the novelty of the food, but increased amounts of eating as the mice became familiar with the task. Indeed, the total food intake over the 3-day period increased as well (**Fig. B3.1**C; main effect of time: $F_{(2,44)}$=91.550, p<0.001). There were still no differences between genotypes in the total amount of food consumed (**Fig. B3.1**C). In summary, we believe that these results indicate a lack of depressive-like behavior in the *Scn1a*$^{RH/+}$ mutants.

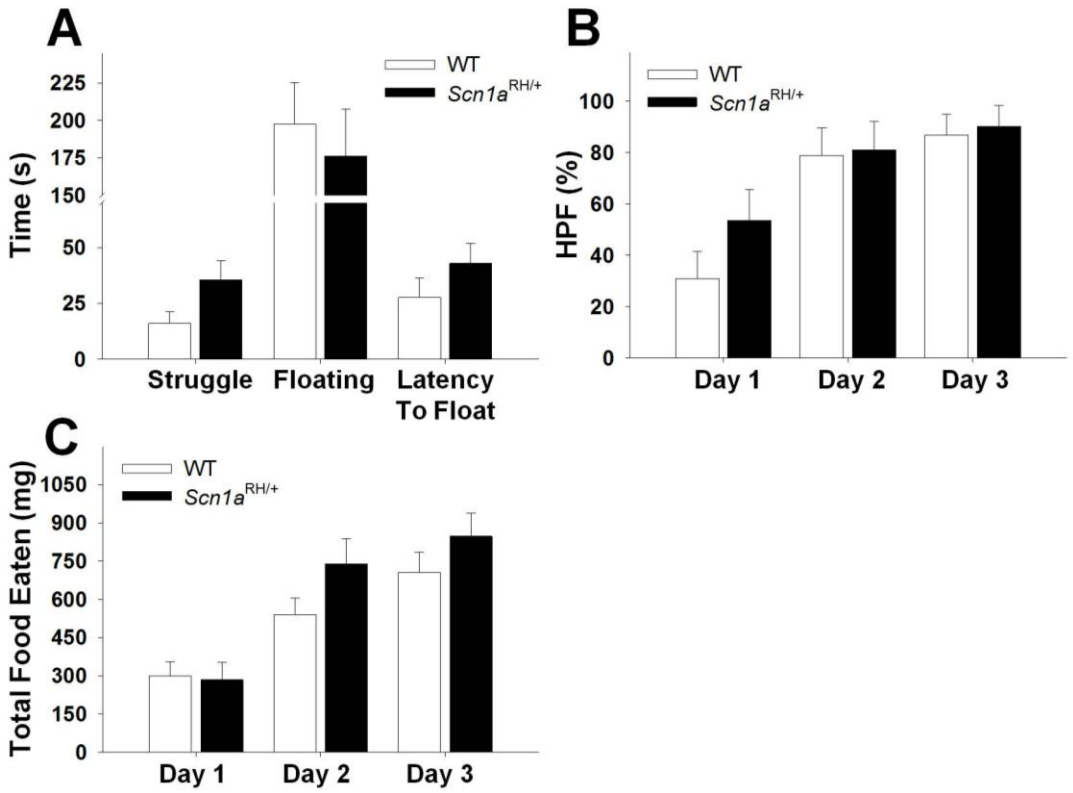

Figure B3.1 *Scn1a*$^{RH/+}$ mutants show no differences in measures of depressive-like behavior. (A) In the forced swim test, there were no differences in the total struggle time, total floating time, or the latency to begin floating between *Scn1a*$^{RH/+}$ mutants and WT littermates. **(B)** In a highly palatable food (HPF) preference paradigm, there were no significant differences in the percentage of food eaten that was HPF between *Scn1a*$^{RH/+}$ mutants and WT littermates, although there was a significant main effect of time ($F_{(2,44)}$=25.445, $p<0.001$) as all mice increased their consumption of HPF over the three day testing period. **(C)** There were no differences in the total amount of food eaten during the HPF preference paradigm between *Scn1a*$^{RH/+}$ mutants and WT littermates, although there was a significant main effect of time ($F_{(2,44)}$=91.550, $p<0.001$), as all groups increased their total consumption over the three day testing period. n=12 all groups. Error bars represent SEM.