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UNIVERSITY OF CALIFORNIA,
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Using Drosophila Models to Understand Patient-Specific Mechanisms of Genetic
Seizure Disorders

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Biological Sciences

by

Alexa Joanna Roemmich

Dissertation Committee:
Professor Diane K. O'Dowd, Chair
Professor Kavita Arora
Professor Todd C. Holmes
Professor Grant MacGregor

2021

DEDICATION

This dissertation is lovingly dedicated to:

My parents, who inspired me in childhood with their own degrees on the wall, and who have given me every opportunity so that I may add my name next to theirs

My grandfather, Ronald Jones, who enthusiastically supported my academic career since I was a little girl, and who I wish were here for the culmination of my schooling

My extended and bonus families, and all the love they have given me throughout the years

And last but not least,

My husband Thomas,   who has supported, encouraged, and loved me in every possible way since we were nineteen. My dreams are easier to achieve with your partnership and I can never thank you enough.

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ACKNOWLEDGEMENTS

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I would also like to thank the other members of my committee, Dr. Todd Holmes, Dr. Grant MacGregor, Dr. Kavita Arora, and Dr. Alan Goldin for their input in the development and completion of these projects.

Lastly, I would like to thank the other members of the O'Dowd Lab for their support and companionship, particularly my primary undergraduate mentee, Thy Theresa Vu, who is a competent scientist in her own right and without whom I would not have completed this work.

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Roemmich AJ, Schutte SS, and O'Dowd DK. (2018). Ex vivo Whole-cell Recordings in Adult Drosophila Brain. *Bio-protocol* 8(14): e2467.

Hartjes KA, Li X, Martinez-Fernandez A, **Roemmich AJ**, Larsen BT, Terzic A, and Nelson TJ. (2014). Selection via pluripotency-related transcriptional screen minimizes the influence of somatic origin on iPSC differentiation propensity. *Stem Cells,* 9:2350-9, 2014.

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Roemmich AJ, Hawkins CC, Sinewitz J, Yu G, Lukacsovich T, Schutte SS, O'Dowd DK. CRISPR-Cas9 knock-in of SCN1A epilepsy-causing mutations, R1648C and R1648H, results in seizure phenotype and altered neuronal firing properties in Drosophila. *Biennial Neurobiology of Drosophila Meeting*, Cold Spring Harbor, October 2017.

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Roemmich AJ, Batts S, Chen Z-Y*.* Isolation of adult mouse inner ear stem cells and progenitors*. Tenth Annual Undergraduate Internship Symposium*, Harvard, Boston, MA August 2014.

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ABSTRACT OF THE DISSERTATION

Using Drosophila to Model SCN1A Epilepsies

by

Alexa Joanna Roemmich

Doctor of Philosophy in Biological Sciences

University of California, Irvine, 2021

Professor Diane K. O'Dowd, Chair

While thousands of epilepsy-causing mutations have been identified throughout the *SCN1A* gene encoding voltage-gated sodium channel Na$_v$1, a protein crucial for regulating neuronal excitability, how most of these mutations lead to seizures is unknown. To effectively treat patients, it is important to understand how individual mutations alter cellular and channel properties and affect the brain. My thesis has focused on two different mutations occurring at one position within *SCN1A*: R1648C associated with the severe disorder Dravet Syndrome, and R1648H, with the milder disorder GEFS+. It is not yet possible to examine cellular activity in the living human brain, so we used genetically modified fruit flies (*Drosophila*) to explore how these two different mutations at the same amino acid location contribute to different behavioral symptoms and diagnostic outcomes.

We used CRISPR-Cas9 gene editing to create Drosophila lines with the R-C or R-H mutation, or R-R control substitution in the fly sodium channel gene *para*. Animals heterozygous for R-C or R-H mutations displayed reduced lifespans, altered circadian behavior and sleep, and spontaneous and temperature-induced seizures not observed in R-

R controls. These behavioral phenotypes, particularly heat induced ("febrile") seizures, are reminiscent of symptoms observed in patients. To examine the underlying cellular mechanisms, we obtained electrophysiological recordings from neurons in the intact brains of adult flies. In GABAergic inhibitory neurons, the R-C and R-H mutants exhibited sustained neuronal depolarizations and altered firing frequency that were exacerbated at elevated temperature. R-H and R-C inhibitory neurons also displayed similar and constitutively hyperpolarized sodium current deactivation thresholds. In contrast, excitatory cholinergic neurons carrying R-C or R-H did not display sustained depolarizations or other obvious differences from controls, implicating decreased inhibition as the main neuronal impact of R1648 mutations. Further, the similarity of the effect of the R-C and R-H mutations in Drosophila with identical genetic backgrounds suggests that genetic modifiers play a crucial role in human presentations of seizure disorders. Finally, this work with *SCN1A* mutant Drosophila was conducive to the creation of a classroom module connecting biological principles used in lecture to real-world research, and resulted in high engagement for both undergraduate students and graduate teaching assistant.

INTRODUCTION

Epilepsy is a broad class of neurological disorders characterized by recurrent seizures, affecting about 1.2% of the US population (Fiest et. al. 2017) and around 50 million people worldwide. Seizure disorders are highly diverse, ranging from nearly undetectable seizure events in some individuals to multiple severe seizures a day in others, greatly decreasing quality of life. Epilepsy can also be associated with a number of comorbidities including ataxia, developmental delay, and sleep disorders, and can even lead to sudden unexpected death in epilepsy (SUDEP) (Devinsky et. al. 2018). Epilepsy disorders are diverse not only in the severity of the seizures that individuals experience but also by the etiology of the disease. Epilepsy etiology can be grouped into three broad categories: 1) symptomatic, resulting from head trauma or structural abnormalities in the brain, 2) provoked, associated with other diseases or environmental factors, and 3) idiopathic, where there is an identified or presumed genetic component (Escayg & Goldin 2010, Devinsky et. al. 2018). This heterogeneity in presentation and cause makes epilepsy a difficult disorder to treat. However, with the great expansion of tools for rapid and accurate genetic sequencing in recent years, there has been significant progress toward an understanding of the connection between genetic epilepsies and disease symptoms.

Epilepsies arising from genetic mutations account for about 30% of all seizure disorders. Since epilepsy is associated with a disruption of balance between excitation and inhibition in the brain, it is not surprising that the majority of mutations occur in genes involved in neuronal excitability. For example, 17% are due to mutations in voltage-gated sodium, potassium, or calcium channels, and 10% more are associated with mutations in

neurotransmitter receptors (Oyrer et. al. 2018). The *SCN1A* gene encoding the alpha subunit of voltage-gated sodium channel Na$_v$1.1 is a particular hotspot, and the most frequently identified gene associated with genetic epilepsy (Menezes et. al. 2020).

Voltage-gated sodium channels open in response to membrane depolarization, allow sodium influx into a cell, and initiate the rising phase of action potentials that propagate electrical signals throughout the nervous system (Hodgkin and Huxley 1952). There are nine different voltage-gated sodium channel pore-forming alpha subunit isoforms in mammals, encoded by the genes SCN1A-SCN5A and SCN8A-SCN11A (Catterall 2010). While all isoforms play important roles throughout the body including in the nervous system, skeletal muscle, and heart muscle, Na$_v$1.1 has been found particularly at the axon initial segments of parvalbumin-positive inhibitory interneurons, suggesting that Na$_v$1.1 may play a particularly important role in modulating network excitability (Ogiwara et. al. 2007, Ravenscroft et. al. 2020).

The Na$_v$1.1 sodium channel, encoded fby *SCN1A*, is composed of four homologous domains (I-IV), each with six transmembrane-spanning segments (termed S1-S6), with S5, S6, and the hairpin loop between them interacting to form the pore and selectivity filter (Fig. 1) (Catterall 2017). The fourth segment of each domain (S4) has conserved positively-charged amino acids every third position, notably arginines, that are important in activating the channel in response to depolarizing membrane potential (Shen et. al. 2017, Pan et. al. 2018).

While epilepsy-causing mutations are common in the voltage-sensing regions there are over 1250 dominantly-inherited mutations identified in patients at locations throughout *SCN1A* that give rise to a spectrum of several different types of epilepsy, including GEFS+

and Dravet Syndrome (DS). GEFS+ is usually a moderate form of epilepsy, associated with 10% of SCN1A mutations and characterized by febrile seizures which persist beyond the age of six. DS is associated with 85% of SCN1A mutations, is generally more severe, and is characterized by autism-like symptoms, delayed development, and impaired motor functions in addition to seizures (Claes et. al. 2009, Lossin 2009). Both types of epilepsy feature spontaneous seizures, as well as febrile, those that can be triggered by hot temperatures or fevers. In addition, patients with these types of epilepsy, particularly those with DS, are frequently resistant to anticonvulsant drugs (Ogiwara et. al. 2007, Escayg and Goldin 2010). Though the loci of these mutations are known, how individual mutations lead to patient symptoms is largely unknown. Furthermore, there exists a great heterogeneity in the penetrance, expressivity, and drug response of patients, even those with the same mutation (Baulac et. al. 1999, Abou-Khalil et. al. 2001, Weber et. al. 2014). Understanding the underlying mechanisms of cellular dysfunction and the connection between genotype and phenotype in SCN1A-mediated epilepsies will be important in improving patient care.

It is not currently possible to study the epilepsy-causing effects of mutations at the individual cell level in living patients. Fortunately, sodium channels are highly conserved and model organisms can be used to elucidate neuronal mechanisms of seizure generation. Beginning in 2001, the labs of Alan L. Goldin and Alfred L. George, Jr. published a series of papers investigating the cellular effects of various point mutations in SCN1A in heterologous expression systems. Studies of isolated sodium currents in oocytes or nonexcitable cell lines found that missense mutations could lead to either sodium current loss of function (decreased/no sodium currents), sodium current gain of function (increased currents or recovery from inactivation), or mixed effects (Escayg and Goldin 2010). While these studies

revealed that sodium channel mutations can lead to varied disease mechanisms, these findings had not been tested in an excitable cell.

The first mouse model of a human SCN1A syndrome was conducted by Yu *et. al.* 2006, in which DS was modelled via knock-down of Scn1a. Homozygous mutants did not survive past postnatal day 15, while heterozygous mutants displayed spontaneous seizures, a lower seizure threshold, and high mortality. Interestingly, sodium currents were selectively reduced in inhibitory hippocampal interneurons but not in excitatory pyramidal neurons, suggesting that a reduction in neuronal inhibition is resulting in over-excitation and seizures in the brain (Yu et. al. 2006). A zebrafish knockout of *scn1Lab*, analogous to the Yu mouse model, also displayed unprovoked seizures, premature mortality, and abnormal electrographic activity (Baraban et. al. 2013). The high vertebrate homology, large brood sizes, and short generation time of zebrafish, combined with the discovery of seizure like behaviors, make the zebrafish a good model for epilepsy studies, particularly for drug screens. The rise of CRISPR/Cas9 technology in zebrafish (Hruscha and Schmid 2015; Hwang et al. 2013) has also helped, and sodium channel studies thus far have been mostly knock-outs (Weuring et. al. 2020).

Precise knock-ins of patient-specific mutations in excitable models are necessary to study how disease phenotypes arise from individual mutations. While over a thousand different mutations were available for research, two mutations of particular interest were R1648H and R1648C in the S4 segment of domain IV (Fig. 1). These mutations occur at one of the gating charges particularly important for activation. Interestingly, although the mutations occur at the same amino acid location within the channel, they lead to different forms of epilepsy. R1648H causes generalized epilepsy with febrile seizures plus (GEFS+) in

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patients (Baulac et. al. 1999), while R1648C leads to Dravet Syndrome (DS) (Ohmori et. al. 2002, Striano et. al. 2008), though the clinical presentations of those affected are highly heterogeneous. The 1648 locus provides an opportunity to study how similar mutations in SCN1A can lead to different behavioral symptoms and diagnostic outcomes.
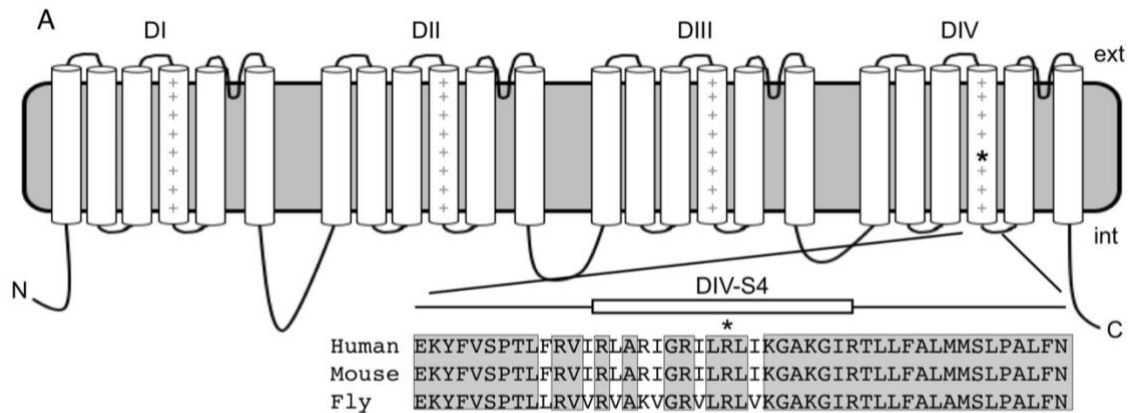
Figure 1. Structure of Nav1.1. The voltage-sensing regions of S4 are shown with +. The black * represents the location of targeted mutations R1648C and R1648H within DIVS4. There is high homology in amino acid sequences between human, mouse, and fly in DIVS4 and connected regions.

The first knock-in models of R1648H and R1648C were in heterologous expression systems and computational models. In those studies, the R1648H mutation resulted in a decreased use-dependence and faster recovery from inactivation. In addition, both R1648H and R1648C mutant sequences resulted in large persistent sodium currents during sustained depolarization not seen in the wildtype channels (Spampanato et. al. 2001, Lossin et. al. 2002, Rhodes et. al. 2004, Vanoye et. al. 2006, Kahlig et. al. 2010). A knock-in mouse model of R1648H demonstrated this mutation resulted in animals that displayed reduced threshold to heat induced seizures. Analysis of isolated neurons indicated decreased repetitive firing selectively in bipolar inhibitory neurons, attributed to reduced sodium currents with slower recovery from inactivation and increased use-dependence inactivation (Tang et. al. 2009, Martin et. al. 2010). Surprisingly, there were no changes in persistent current levels, which

had been a key result from heterologous expression system studies. These findings demonstrate that a single mutation can result in distinct underlying mechanisms of cellular dysfunction in heterologous expression systems and excitable cells. However, since there is no R1648C mouse model to date, it has not been possible to explore similarities and differences in changes associated with R-H vs R-C in mouse neurons. In addition, it is unknown how temperature affects channel function for either mutation.

One avenue to pursue this research is in induced pluripotent stem cell (iPSCs) derived neurons carrying patient-specific mutations. Different DS-encoding mutations have been studied in a few iPSC-derived neuronal models, resulting in hyperactivity due to either impaired excitability in inhibitory neurons (Higurashi et. al. 2013, Liu et. al. 2013, Sun et. al. 2016) or increased excitability in excitatory neurons (Jiao et. al. 2013). A GEFS+ iPSC-derived neuronal model of the K1270T *SCN1A* mutation was recently found to reduce inhibition in inhibitory neurons, resulting in a hyperexcitable network (Xie et. al. 2019). This study also crucially used isogenic pairs of iPSCs to examine the relationship between mutation and disease mechanisms independent of genetic background (Xie et. al. 2019). This work could be used for other mutations, including R1648H and R1648C. Our lab generated homozygous R1648C iPSC-derived neurons and unexpectedly demonstrated that excitatory neurons with the mutation had decreased excitatory firing (Parampathu and O'Dowd 2020). Future studies should investigate R1648C inhibitory neuron phenotypes.

Though iPSC-derived neurons provide an invaluable human context, the model lacks the advantages of whole organisms, namely behavioral phenotypes. To evaluate the effects of multiple mutations at a single locus on organismal and neuronal behavior, and to explore

the effects of temperature on both cellular and behavioral phenotypes, we used a Drosophila model. One advantage of using Drosophila is that they have only a single sodium channel gene as compared to mammals that have multiple genes encoding voltage-gated sodium channels, including three that are highly expressed in the adult brain (*SCN1A*, *2A* and *8A*) (Escayg and Goldin 2010). The Drosophila voltage-gated sodium channel gene (*para*) encodes a protein (Para) similar in structure and function to $Na_V1.1$ (O'Dowd et. al. 1989, Loughney et. al. 1989). The first known *para* mutants, *para^{ts1}* and *para^{bss1},* were identified by forward genetic screens and resulted in heat-induced paralysis (Siddiqi and Benzer 1976; Suzuki et al. 1971) and mechanically-induced seizures (Ganetzky and Wu 1982, Parker et. al. 2011), respectively. These foundational mutants have been invaluable for the study of sodium channels and seizure disorders in Drosophila, however, they did correspond to known epilepsy-causing mutation in humans.

Our lab previously used flies created by homologous recombination to evaluate the first knock-ins of epilepsy in Drosophila. Knock-in Drosophila homozygous for the *SCN1A* mutation K1270T causing GEFS+ or S1231R causing DS displayed heat induced seizure phenotypes with distinct properties in the two mutant lines that were not seen in the controls (Sun et. al. 2012, Schutte et. al. 2014, Schutte et. al. 2016). It was also noted that the mutations caused distinct alterations to sodium channel activity in homozygous neurons. K1270T, causing GEFS+, led to a gain-of-function in sodium channel activity (channel over-activated), while S1231R, which causes DS, led to a loss-of-function in sodium channel activity (channel under-activated). Since distinct behaviors in humans corresponded with distinct behavioral and mechanistic phenotypes in flies as well, we are interested in the

possibility of using many mutant *Drosophila* lines to further model and understand different patient-specific epilepsy-causing mutations.

The goal of generating many Drosophila lines carrying different mutations became easier with the use of CRISPR/Cas9 gene editing. Individual point mutations can be precisely and swiftly targeted without the insertion of a cassette, and the same gene editing tools can be used for thousands of mutations. In the present study, a two-step CRISPR-Cas9 gene editing process was used to insert either R1648C, R1648H, or R1648R into the Drosophila sodium channel gene. This process was highly efficient and precise. Mutant lines were created carrying no additional mutations, demonstrating proof-of-concept. This two-step method can be used in the future to quickly generate lines for the other SCN1A mutations in the same exon. Studying R1648C and R1648H in Drosophila after studying previous Drosophila models K1270T and S1231R allows us to compare two DS mutants and two GEFS+ mutants. Some of the questions that can be addressed include determining whether underlying mechanisms of disease are more similar between mutations associated the same epilepsy diagnosis, or between those with different mutations at the same locus.

Heat-induced seizure, lifespan, and activity analyses were conducted on mutant Drosophila. Whole-cell patch clamp electrophysiology was performed on inhibitory and excitatory cells in the adult antennae lobes. Whole-brain dissections and the electrophysiological process were described in updated detail for increased replicability (Roemmich et. al. 2018). R1648H and R1648C flies displayed similarly high seizure susceptibility, reduced lifespan, and altered circadian activity. R1648H and R1648C flies also displayed similar occurrences of heat-exacerbated sustained depolarizations and

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constitutively hyperpolarized sodium current deactivation thresholds in inhibitory neurons. Diversity seen in human patients was not observed in the Drosophila lines with different mutations at the same amino acid position in the gene within identical genetic backgrounds, suggesting that genetic modifiers at least at the R1648 locus play a crucial role in human presentations of seizure disorders.

As a scientist-educator, I am also interested in finding ways to effectively engage college biology students with authentic research as a way to encourage and inspire them to think more deeply about where scientific knowledge comes from and how it can be used. Therefore I developed a module to bring the Drosophila seizure models I created as part of my thesis work into an introductory biology discussion section. The classroom module using our *SCN1A* Drosophila was memorable for students as it led to an increased connection between biological principles learned in lecture and used in real-world research, and it resulted in an increase in their interest in on-campus research. Using thesis research in a teaching assistant role also taught me how to structure a lesson, incorporate many teaching objectives into one lesson, and built confidence in leading a classroom.

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CHAPTER II

Ex Vivo Whole-Cell recordings from Adult Drosophila Brains

This work has been published in Roemmich AJ, Schutte SS, O'Dowd DK (2018) Ex vivo Whole-cell Recordings in Adult Drosophila Brain. *Bio-protocol* 8(14): e2467.

2.1 Introduction

The fruit fly (*Drosophila melanogaster*) has been used to make key discoveries in a variety of fundamental areas in neuroscience including learning and memory (Bolduc *et. al* 2008, Cervantes-Sandoval *et. al*. 2016), synapse formation and regulation (Genç *et. al.* 2017), and circadian rhythms (Allada *et. al.* 1998, Guo *et. al.* 2016). Mutants identified through both forward and reverse genetic screens have also provided useful models of human neurological disorders including Fragile X syndrome, Parkinson's Disease, Huntington Disease and epilepsy disorders (Sears & Broadie 2018, Liu *et. al.* 2012, Pallos *et. al.* 2008, Parker *et. al.* 2011). Much of what we have learned in this research comes from electrophysiological recordings and calcium imaging at the neuromuscular junction (NMJ), from neurons in dissociated primary culture, or from the central nervous system of embryos and larvae. Though these methods have been instrumental in our understanding, to elucidate the underlying cellular mechanisms of neurological processes in adult animals, it is important to have electrophysiological access to individual neurons of the adult brain.

Recordings from neurons in the adult CNS were made possible by development of two complementary systems in the mid-2000s. One involves exposing and desheathing a small area of brain making it possible to obtain intracellular recordings from neurons in a live, behaving adult fly (Wilson, Turner, & Laurent 2004, Nagel & Wilson 2016, Hige *et. al.* 2015). This preparation is best suited to recording from populations of neurons on the dorsal surface of the brain. The second preparation involves removing the whole brain from the adult head capsule and placing it in a recording chamber (Gu & O'Dowd 2006, 2007). This provides access to neurons in the entire brain and allows for easy environmental manipulations. Although the process is invasive and may cause damage to the brain, intact

neurons and functional circuits can be persevered and maintained for up to one hour after skillful dissection. Labs have used whole brain dissection and whole-cell recordings to characterize the electrical properties of circadian neurons (Sheeba *et. al*. 2008a), uncover the electrical cellular mechanisms responsible for sleep and arousal (Sheeba *et. al.* 2008b), discover a new light-sensing pathway in the brain (Ni *et. al.* 2017), determine the mechanism of action for a common pesticide (Qiao *et. al*. 2014), find a memory suppressor miRNA that regulates an autism susceptibility gene (Guven-Ozkan *et. al.* 2016), and describe synaptic dysfunction in a model of Parkinson's Disease (Sun *et. al.* 2016).

Our lab uses this protocol extensively to study the cellular mechanisms of genetic epilepsy associated with mutations in SCN1A, a gene that encodes NaV1.1 sodium channels that are highly expressed in inhibitory, GABAergic neurons in the human brain. Using homologous recombination, and more recently CRISPR/Cas9 mediated gene editing, we have introduced specific SCN1A missense mutations into the same location in the *Drosophila* sodium channel gene, para. We have shown that all of the mutations causing febrile seizure phenotypes in humans that we have examined (K1270T, S1231R, R1648H/C), also result in heat-induced seizure phenotypes in the adult fly (Sun *et. al.* 2012; Schutte *et. al.* 2014; Schutte *et. al.* 2016). To evaluate how specific mutations alter sodium currents and neuronal activity, we perform electrophysiological analyses of sodium currents in knock-in flies carrying SCN1A mutations, focused primarily on GABAergic, local neurons (LNs) in the antennal lobe. Whole-cell recordings from the cell bodies of LNs can be used to evaluate sodium currents and firing properties in mutant compared to wildtype neurons. The ability to rapidly exchange extracellular recording solutions in the *ex vivo* preparation allows fast and reversible elevation of the temperature to assess constitutive and temperature-

dependent changes in sodium currents and firing properties in knock-in mutant compared to wildtype neurons. Fast perfusion also facilitates evaluation of the acute effects of potential anti-convulsant drugs on sodium currents and firing properties. Here we present our updated protocol for *ex vivo* whole-cell recordings in adult *Drosophila* brains, including fly dissection and preparation, data acquisition, and analysis.

2.2 Methods

Ex Vivo Preparation

Whole-brain dissection from adult fly

Video of this section is available on Jove (Gu & O'Dowd 2007).

1. Prepare dissecting solution (see the recipe below). The dissecting solution is best used within 4-5 hours after preparation.

2. Put fly vial on ice for ~1 minute to anesthetize flies (watch carefully- it will only take a short time to slow the flies down, and too long on ice will kill them).

3. Place small drop (~60μl) of dissecting solution in lid of 35mm Petri dish.

4. Use a pair of fine-tip tweezers and the dominant hand to pick up an anesthetized fly by the wings or legs.

5. With the non-dominant hand, use syringe needle to pin down thorax onto the Petri dish (NOT into the dissecting solution).

6. Take a second syringe needle with the dominant hand and decapitate fly.

7. Stabilize hands by resting them on microscope stage, and keep fingers close to the needle tips. With head facing up, pin down the fly's left eye with the needle in the non-dominant hand (needle 1). With needle in the dominant hand (needle 2),

make a vertical cut just medial to needle 1 that extends from the rostral to caudal surface (Fig. 1).

Steps 6-8

Cut eye

Mouthparts

= 0.5mm

Figure 1. Cutting the cuticle. Pin down each eye and cut off the outer portion. Then pin and cut away the mouthparts. The cuticle will now be open on three sides to dissecting solution.

8. Turn brain 180 degrees. Pin down the fly's right eye with needle 1, and again with needle 2 make a vertical cut just medial to needle 1 that extends from the rostral to caudal surface (Fig. 1).

9. Pin down mouthparts with needle 1 and with needle 2, make a horizontal cut that extends the entire width of the fly head and bisects the cut edges of the eyes (Fig. 1).

10. Carefully push the cut head into drop of dissecting solution and set a timer for 5 minutes.

11. Turn over the fly head so that the rostral side is on the dish. Pin down the bottom edge of the rostral cuticle, beneath the brain, with needle 1, and use needle 2 to open "the flap of" the caudal cuticle like a book (Fig. 2A).

12. The intact fly brain should now be exposed. Still pinning down the rostral cuticle with needle 1, use the back side of needle 2 to gently push the brain out of the head capsule (Fig. 2B).

13. Clean the brain by pinning down connective tissue with needle 1 and gently pushing the brain away with needle 2. As much connective tissue should be removed as possible (Fig. 2C).

14. Entire dissection process should take less than 10 minutes.

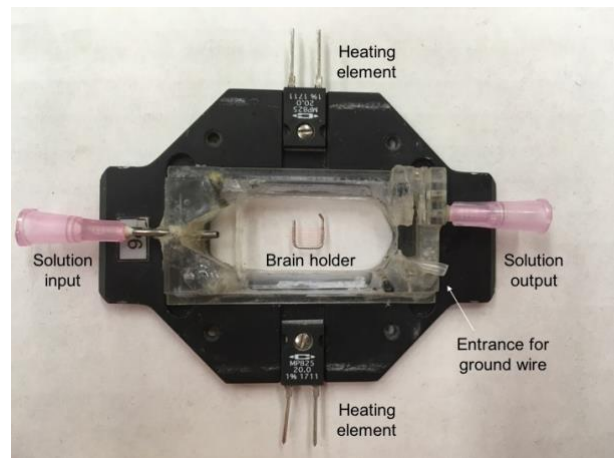**Figure 2. Removing the brain from the cuticle**. A. With the caudal cuticle face-up, use needle 1 to pin down the underhanging tab of the rostral cuticle. Use the backside of needle 2 to open the caudal cuticle like a book. B. Still pinning the inner surface of the rostral cuticle with needle 1, use the backside of needle 2 to push the brain out of the head cuticle. C. Clear off connective tissue to end with a clean and intact *Drosophila* brain. Clean brain imaged with compound microscope with rostral side up. Under dissecting microscope, brain will look milky gray with bright white connective tissue.

Mounting fly brain for electrophysiology

1. Fill recording chamber with external solution. We use a chamber and platform set that allows for solution perfusion as well as heating of the chamber (Fig. 3).

2. Transfer the brain to recording chamber using a 200ul pipet. Note that the fly brain may get stuck on the inside of the pipet tip especially if the connective tissue was not fully cleaned off the brain.

Figure 3. Recording chamber and holder. The chamber is connected to a perfusion system on either side to keep solution flowing. The chamber also contains connections to a temperature controller that allows us to heat the chamber directly to warm solution surrounding the brain. A brain holder (described in mounting step 4) secures the brain for recording.

3. Use a needle to gently push the brain to the desired position. For most of our experiments, we position the caudal part of the brain on the chamber surface with the

antennal lobes facing up. The position may be changed based on the specific need of different experiments.

4. Stabilize the brain using a brain holder as shown in Fig 4. The brain holder is made in our lab. The frame is made with a platinum wire with dimensions of 6mm x 6mm. The nylon fibers are glued on to the frame with super glue and the distance between each fiber is about 0.5-1mm to accommodate the variation in brain size (but most intervals between fibers are closer to 0.5mm). To best stabilize the brain, the fibers should cross the brain at the junction between the optic lobes and central brain region (Fig. 4).
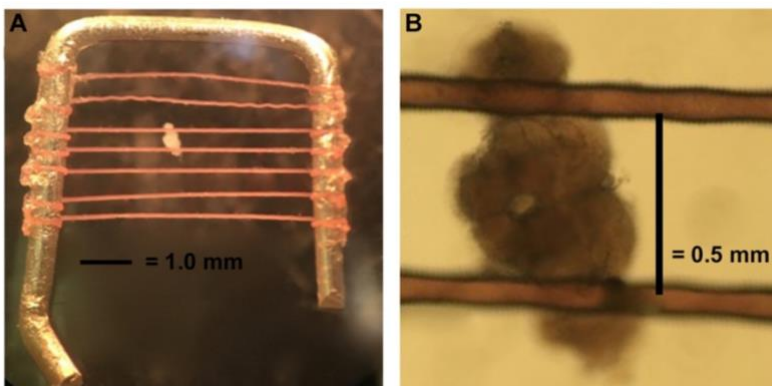
Figure 4. Mounting the brain.

A. The brain holder is made of a bent platinum wire crossed by horizontal fibers a little less than 0.5 mm apart. B. Orient the dissected brain as shown and hold in place with platinum holder.

Whole-cell Recording

Our headstage is on the right of our rig setup and we use borosilicate glass micropipettes (100µl) that are pulled on a micropipette puller in two stages: final tip resistance of 9-10Ω.

1. Circulate external solution with a perfusion (peristaltic pump) system. Oxygenation of the external solution is not necessary when it is constantly perfused at low speed. No minimal speed has been established, but we generally keep the perfusion at 0.8ml/min for our recording chamber which holds 1-1.5ml external solution. The

circulating speed may vary depending on experimental needs, for example, fast drug administration or temperature change may require more rapid perfusion. However, perfusion that is too rapid may generate electrical noise or flush the brain out of place.

2. Fill a pipette with internal solution and load onto headstage. Apply positive pressure to keep the pipette tip free of debris.

3. Use manipulator to bring pipette above brain and slightly to the right.

4. Local neurons are suspended above the neuropil area of the antennal lobes. As pipette approaches LNs, cells should move away slightly from pipette (Fig. 5). For those who wish to investigate excitatory neurons, cholinergic projection neurons (PNs) are located medially on the antennal lobe. The spikelet and current amplitudes of this population can be quite small. On the ventral side of the brain, large and small ventral lateral neurons implicated in circadian rhythms and sleep have also been studied using this protocol.

5. Release pressure on cell moving away to form a giga-ohm seal. Then break into the cell to record.
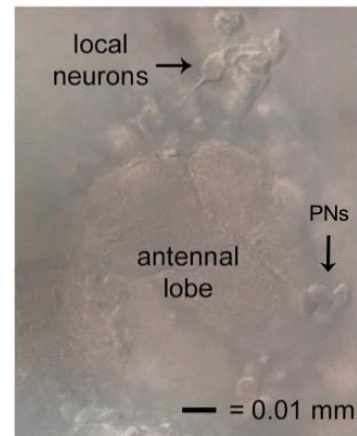
Figure 5. Locating local neurons. Local neurons good for recording will be suspended above, but tethered to, the neuropil of the antennal lobe, have smooth cell membranes, and move away slightly when approached. The excitatory neurons of this circuit, the projection neurons (PNs), also shown.

Osmolarity is crucial to the success of patches and recordings. If cells instantly break in without forming a giga-ohm seal, the osmolarity difference is too large. If cells don't move away from the pipette or can't be broken into, the osmolarity difference is too small. External solution should be 15-20 mOsm L^{-1} greater than internal solution. We use a vapor pressure

osmometer to approximate, but adjustments are common. A 3% dilution decreases osmolarity by about 10 mOsm L^{-1}. We recommend keeping the internal solution in a small sealed container, such as 1.5 ml centrifuge tubes. If the internal solution in an open container, the osmolality can change drastically (20-30 mOsm L^{-1}) in a few hours due to water evaporation.

2.3 Data Analysis

Record and analyze traces using the pClamp suite. Sample traces for our protocols are shown below (Fig. 6-8). More details regarding data processing and analysis can be found in Sun *et. al.* 2012, Schutte *et. al.* 2014.
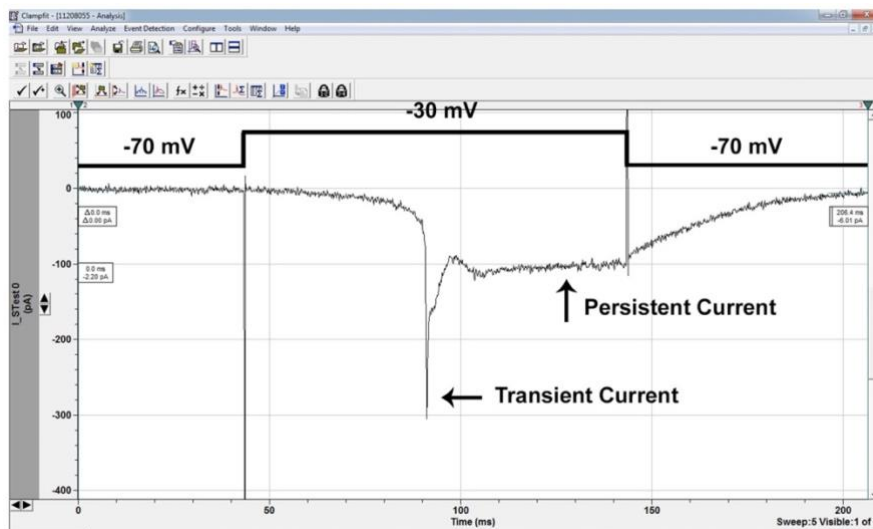
Figure 6. Sodium currents. Sodium current in LN in response to depolarizing voltage-step as shown. Sodium currents are not well space-clamped as the sodium channels are located on the processes, not the cell body we record from, and this is also responsible for the delay between the depolarizing step and the onset of channel opening. In this trace, the cell was held at -70mV and a series of depolarizing traces applied to activate the channel. -30mV was the first sweep to activate the currents, and is characterized as the activation threshold. The transient current amplitude is about 300pA, and the persistent current amplitude is about 100pA in this case. Mutant cells may have activation thresholds and/or current amplitudes greater or lower than wildtype cells. The channel deactivates after the stimulus step has ended, a phenotype that is sometimes impaired in mutant cells. Voltage sweeps can be applied after channel activation to investigate channel deactivation threshold as well.
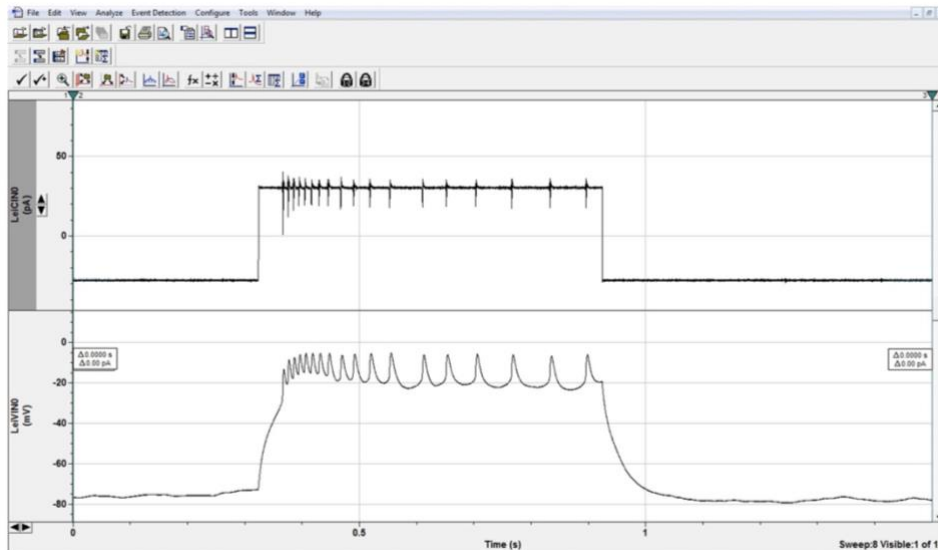
Figure 7. Evoked Firing. Trains of action potential spikelets in LNs in response to depolarizing current step as shown. In current clamp one can study the evoked firing properties of neurons, including spikelet amplitude, and spikelet frequency as a function of stimulus strength. This wildtype neuron fired 18 spikelets in response to a 30pA stimulus. Higher or lower spikelet frequency in mutant neurons compared to frequency in control cells indicates hyper- or hypoexcitability, respectively. The amplitude peaks are smaller than those seen in other organisms as we are recording from the inexcitable cell soma and action potentials are generated on the distant processes.
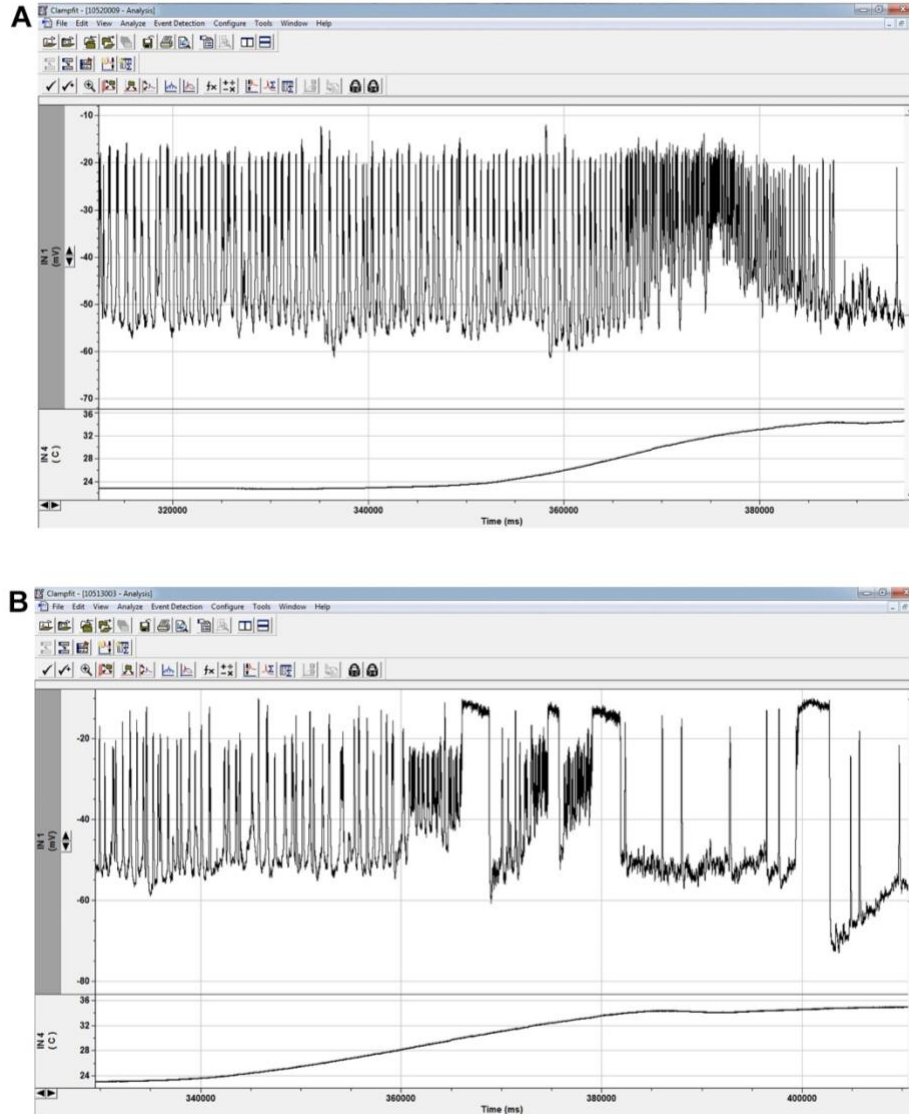
Figure 8. Spontaneous firing and heating protocol. Spontaneous firing recording in control (A) and mutant (B) LNs as temperature is elevated from 22°C to 35°C. Due to our *ex vivo* preparation, we can run any protocol under different conditions. Since our lab studies mutant lines with temperature-sensitive phenotypes, recordings at high temperature are imperative for our understanding of the channel and cellular consequences of channel mutations. Major trace aspects we analyze are spike and burst thresholds, frequencies, and durations. Wildtype cells exhibit increased firing at elevated temperatures (A). The sustained depolarizations in mutants (B) were largely a heat-sensitive phenotype and indicated a gain-of-function mutation for sodium channel activity (Schutte *et. al.* 2016). (Additional equipment: Harvard Apparatus In-Line Solution Heater, Model: SHM-828, and Harvard Apparatus Temperature Controller, Model: CL-100)

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CHAPTER III

Seizure phenotype and underlying cellular defects in Drosophila knock-in models of DS (R1648C) and GEFS+ (R1648H) SCN1A epilepsy

3.1 **Introduction**

Mutations in voltage-gated sodium channels are implicated in a range of human seizure disorders (Escayg and Goldin 2010). In the sodium channel gene *SCN1A,* there are over 1250 dominantly-inherited mutations identified in patients with epilepsy. Though the loci of these mutations are known, how individual mutations lead to patient symptoms is largely unknown.

The $Na_V1.1$ sodium channel, encoded for by *SCN1A*, is composed of four homologous domains (I-IV), each with six transmembrane-spanning segments (termed S1-S6) (Catterall 2017). The fourth segment of each domain (S4) has conserved positively-charged amino acids every third position, notably arginines, that are important in activating the channel in response to depolarizing membrane potential (Shen et. al. 2017, Pan et. al. 2018). The S4 segment in domain IV is unique in that its movement is slower, and the movement uncovers a binding site for the inactivation loop in the pore (Chen et. al. 1996, Chanda and Bezanilla 2002, Goldschen-Ohm et. al. 2013). In this study, we focused on two mutations in the S4 segment of domain IV, R1648H and R1648C. Although the mutations occur at the same amino acid location within the channel, they lead to different forms of epilepsy. R1648H causes generalized epilepsy with febrile seizures plus (GEFS+) in patients (Baulac et. al. 1999), while R1648C leads to Dravet Syndrome (DS) (Ohmori et. al. 2002, Striano et. al. 2008). GEFS+ is usually a moderate form of epilepsy, characterized by febrile seizures which persist beyond the age of six. DS is generally more severe and is characterized by delayed development and impaired motor functions in addition to seizures. Both types of epilepsy feature febrile as well as spontaneous seizures. In addition, patients with these types of

epilepsy are frequently resistant to anticonvulsant drugs (Ogiwara et. al. 2007, Escayg and Goldin 2010). As the connection between individual mutations and patient symptoms remains largely unknown, we aim to model patient specific epilepsies in the lab.

Studies of isolated sodium currents in oocytes, HEK cells, and computational models found that the R1648H mutation resulted in a decreased use-dependence and faster recovery from inactivation. In addition, both R1648H and R1648C mutant sequences resulted in large persistent sodium currents during sustained depolarization not seen in the wildtype channels (Spampanato et. al. 2001, Lossin et. al. 2002, Rhodes et. al. 2004, Vanoye et. al. 2006, Kahlig et. al. 2010). Later studies using a knock-in mouse model of R1648H demonstrated this mutation resulted in animals that displayed reduced threshold to heat induced seizures. Analysis of isolated neurons indicated decreased repetitive firing selectively in bipolar inhibitory neurons, attributed to reduced sodium currents with slower recovery from inactivation and increased use-dependence inactivation (Tang et. al. 2009, Martin et. al. 2010). Surprisingly, there were no changes in persistent current levels, which had been a key result from heterologous expression system studies. These findings demonstrate that a single mutation can result in distinct underlying mechanisms of cellular dysfunction in heterologous expression systems and excitable cells. However, since there is no R1648C mouse model to date, it has not been possible to explore similarities and differences in changes associated with R-H vs R-C in mouse neurons. In addition, it is unknown how temperature affects channel function for either mutation.

In order to evaluate the effect of the same mutation at a single locus in excitable cells, and to explore the effects of temperature on both cellular and behavioral phenotypes, we

used? the Drosophila model. One advantage of using Drosophila is that they have only a single sodium channel gene as compared to mammals that have multiple genes encoding voltage-gated sodium channels, including three that are highly expressed in the adult brain (*SCN1A*, *2A* and *8A*) (Escayg and Goldin 2010). The Drosophila voltage-gated sodium channel gene (*para*) encodes a protein (Para) similar in structure and function to $Na_V1.1$ (O'Dowd et. al. 1989, Loughney et. al. 1989). Previous studies showed that knock-in animals homozygous for the *SCN1A* mutation K1270T causing GEFS+ or S1231R causing DS displayed heat induced seizure phenotypes with distinct properties in the two mutant lines that were not seen in the controls (Sun et. al. 2012, Schutte et. al. 2014, Schutte et. al. 2016). It was also noted that the mutations caused distinct alterations to sodium channel activity in homozygous neurons. In the present study, CRISPR-Cas9 gene editing was used to insert either R1648C, R1648H, or R1648R into the Drosophila sodium channel gene. Behavioral and electrophysiological studies were performed on R-C and R-H heterozygous mutants and R-R controls.

3.2 Materials and Methods

Drosophila Generation

A plasmid expressing the DsRed marker fused to an eye-specific promoter and flanked by worm sequences and two arms (2 and 1.4kb) homologous to *para*, as well as plasmids expressing gRNA, were injected into fly embryos that expressed Cas9 under the germ-cell promoter *vas*. Successfully transformed adults were selected based on DsRed expression in their eyes. *Para* is located on the X chromosome in *Drosophila*, so disruption of the *para* gene in DsRed flies resulted in lethality in hemizygous males and homozygous females. The line was maintained by balancing the mutant X chromosome with FM7.

In the second step, a plasmid that expresses gRNA targeted to the worm sequences flanking DsRed, along with a plasmid containing the *para* gene exon 30 containing a point mutation, were injected into *vas*-Cas9 embryos. The repair template carried at the 1648 amino acid codon position either the desired mutation R-C (CGA to TGT), R-H (CGA to CAT), or a silent mutation R-R (CGA to CGT) that serves as our control. Injected fly embryos that matured may produce eggs with the restored but mutated *para* gene. Females were crossed with FM7/Y males.

For the R-C and R-R transformation, the presence of the mutated *para* gene rescued male lethality, so males with non bar-eyes (expressing mutated *para* on the manipulated X chromosome) were used as founders for line propagation, and then sequenced for genotype verification. For the R-H transformation, male lethality was not rescued. Female flies were screened for the loss of DsRed, and then used as founders and sequenced. Sequencing of ~1.7 kb of DNA surrounding the targeted mutation site and gRNA cut sites confirmed the presence of the predicted base pair change in each line with no additional mutations, other than a few silent mutations where two different codons for the same amino acid were present in the population. Multiple, independent lines were obtained, backcrossed for five generations on a UAS-GFP; w+ background, and maintained. R-C and R-H mutations are homozygous lethal so all lines are maintained as heterozygous stocks over the X-chromosome balancer FM7.

Seizure Behavior Test

Flies one day post-eclosion were placed in groups of five in empty vials that were then immersed in a water bath, first at room and then at high temperature for two minutes. Video recordings of the assay were evaluated and the number of flies seizing in each vial at 20

second intervals were determined. A seizure was defined as a fly exhibiting a whole-body twitch or jump, or falling onto its back or side, often accompanied by leg twitching or wing flapping. Researchers were blinded to genotype during analysis. Data from multiple experiments were used to determine the average probability of seizure for a fly of a certain genotype at any second during the assay.

Lifespan Assay

Three groups of 10 newly eclosed female flies from both mutant lines, R1648C and R1648H, as well as the control line R1648R, were separated by genotype and placed in vials laid horizontally containing standard cornmeal-based food. The flies were housed under a 12 hour light, 12 hour dark cycle at 23ºC. Fatalities were recorded every 1-3 days and food vials were changed twice/weekly. Survival curves were created using the Kaplan-Meier estimate, and analyzed using the Mantel-Cox logrank test.

Electrophysiology

Whole brain dissections were conducted on adult female mutant or control flies two-days post eclosion. Briefly, the fly was anesthetized on ice for one minute, then decapitated. The eyes were cut and mouth parts removed, and the rest of the head was placed into an external solution (described below) also containing a papain suspension. After five minutes, the brain was removed from the cuticle and connective tissue removed. A dorsal lateral subset of inhibitory local neurons were targeted in anatomically distinct regions of the antenna lobes.

Whole-cell sodium currents and depolarization-evoked action potentials were recorded with whole cell pipettes of 9-11 MΩ. Sodium currents were recorded using a pipette

solution containing (in mM) 102 CsOH, 102 D-gluconic acid, 17 NaCl, 0.085 CaCl$_2$, 1.7 MgCl$_2$, 8.5 HEPES, 0.94 EGT, and 25 ATP. The pH was adjusted to 7.2 with CsOH and the osmolarity adjusted to 232-230 mOsm. The external solution contained (in mM) 122 NaCl, 3.0 KCl, 1.8 CoCl$_2$, 0.8 MgCl$_2$, 5.0 glucose, and 10 HEPES. It also contained 2.5mM of tetraethylammonium (TEA), 1.0mM 1-aminopyridine (4-AP), 20µM (+)-Tubocurarine, and 10µM of Picrotoxin (PTX). The pH was adjusted to 7.2 with NaOH and the osmolarity adjusted to 250-255 mOsm L^{-1}. Evoked action potentials were recorded using the same internal solution, replacing the CsOH with 102mM potassium gluconate and the D-gluconic acid was removed. The external solution was the same except the CoCl$_2$ was replaced by CaCl$_2$ and the TEA and 4-AP was removed. Spontaneous action potentials were recorded using the same internal and external solutions as for evoked action potentials, without the presence of blockers. The chamber was continuously perfused at 0.75 – 1.0 ml/min and the temperature in the chamber was controlled and monitored using a CL-100 Bipolar Temperature Controller (Harvard Apparatus). Data were acquired with an Axopatch 200B amplifier (Molecular Devices), a Digidata 1322A digital-to-analog converter (Molecular Devices), a Dell computer (Dimension 8200), and pClamp9 software (Molecular Devices).

Statistics

Statistical significance was determined using ANOVA with Tukey's post hoc test for multiple experimental groups. Paired t-tests were used to determine significance between room and high temperature data within a single genotype. Data is shown as mean +/- standard error.

3.3 Results

CRISPR-Cas9 generation of Drosophila carrying epilepsy-causing mutations

To create flies expressing patient-specific epilepsy-causing mutations, a two-step CRISPR-Cas9 mediated genome editing strategy was employed. In the first step, exon 30 of the *para* sodium channel gene, where the target missense mutations are located, was replaced
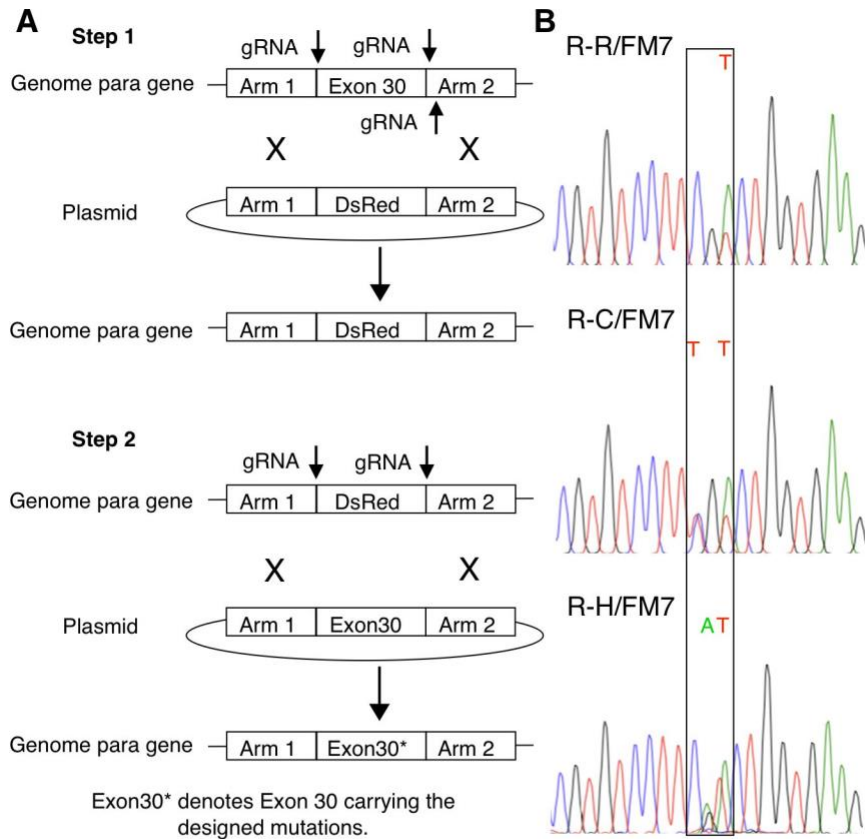
Figure 1. CRISPR strategy and validation. A. Scheme of the 2-step CRIPSR-Cas9 mediated genome editing for targeting the mutations to the Drosophila para gene. B. Mutant Drosophila lines were confirmed by Sanger sequencing. The wild type codon is CGA. Step 2 repair plasmids carried the silent mutation CGT for R-R controls, TGT for R-C mutants, and CAT for R-H mutants.

by a DsRed marker (Fig. 1A). In the second step, the DsRed marker was replaced by exon 30 of the para gene containing either the desired mutation R-C (CGA to TGT), R-H (CGA to CAT), or a silent mutation R-R (CGA to CGT) that serves as the control (Fig. 1A). Sequencing of ~1.7 kb of DNA surrounding the targeted mutation site and gRNA cut sites (Fig. 1B) confirmed the presence of the predicted base pair change in each line with no additional mutations. Multiple, independent lines were obtained, each resulting from a unique CRISPR insertion of R-C, R-H, or R-R. Both R-C and R-H mutations are homozygous lethal so all lines were maintained as heterozygous stocks over the X-chromosome balancer FM7. Patients with

these mutations are heterozygous so all experiments were performed on heterozygous females.

Mutants display seizure-like behavior and reduced lifespan

Three fly lines heterozygous for the R-C mutation and four lines heterozygous for the R-H mutation were monitored for presence of spontaneous and/or heat-induced seizure behavior. Groups of five flies were collected one day post-eclosion and placed in a vial. A two-minute video was recorded immediately following immersion of the vial into a water bath, first at room (22ºC) and then at high temperature (40ºC). Videos were later evaluated for seizure activity. A seizure was defined as the fly exhibiting a whole-body twitch or jump, or falling onto its back or side, often accompanied by leg twitching or wing flapping. Analysis was conducted blinded with respect to genotype.

Results are reported as the average probability of seizure for a fly of a certain genotype at any point in time during the assay. The four R-R control fly lines did not exhibit seizure activity in water baths at room temperature and very minimal seizure activity at high temperature (Fig. 2A). In contrast, both R-C and R-H heterozygous flies had a significantly increased seizure probability at room temperature compared to the R-R/FM7 control. At high temperature seizure probability increased significantly in both R-C and R-H when compared to their behavior at room temperature, and to control R-R flies at high temperature (Fig. 2A, $p < 0.05$, ANOVA, Tukey's *post hoc* test). There was no difference in seizure probability between lines with the same mutation. These data indicate that the seizure activity is associated with the introduced mutations, either R-C or R-H, and is not

influenced significantly by the specific insertion events during the CRISPR process that resulted in generation of each line.

To evaluate the time course of heat induced seizure activity associated with the different mutations, multiple flies in single R-H, R-C and R-R lines were examined at 20 second intervals over a two-minute period following immersion of the vial in water bath of 40ºC. The actual temperature inside the vial was continuously monitored during heating, reaching a temperature of 33°C at 2 minutes (Fig. 2Bi). The onset of heat induced seizures was similar in the R-C and R-H lines, with maximal seizure probability occurring at 120 seconds (Fig. 2B). The similarity in the spontaneous and heat induced seizure phenotypes in the R-C and R-H lines
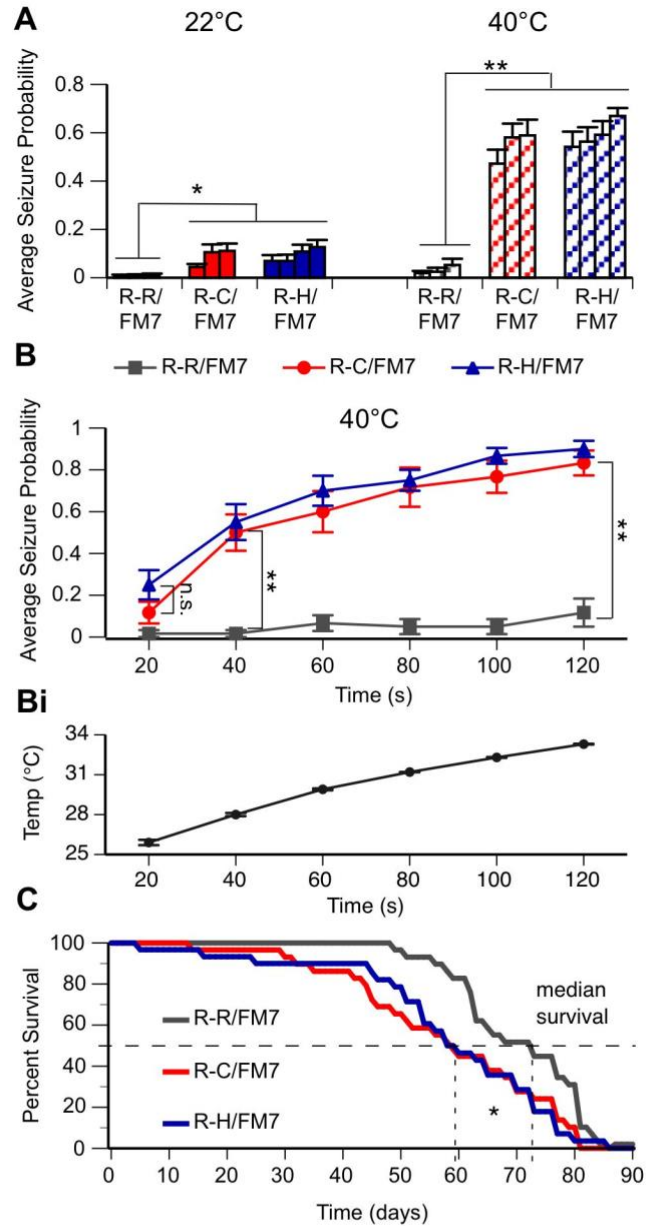
Figure 2. R-C and R-H mutants display seizure-like behavior and reduced lifespan. (A) Average seizure probability at any point in time in a two-minute trial for each independent line (3 R-R/FM7, 3 R-C/FM7, 4 R-H/FM7). (B) Average seizure probability for each genotype shown at 40ºC over time. At both 22ºC and 40ºC, R-C and R-H mutants have a significantly greater average seizure probability compared to R-R controls. Comparisons between R-C and R-H flies or between lines of one genotype are not significant * $p<0.05$, ** $p<0.01$. Two-way ANOVA, Tukey's post-hoc test. $n > 12$ trials per group. (Bi) Average temperature inside vial over time of vial in 40°C bath. (C) Kaplan-Meier survival curve estimates of R-R, R-C, and R-H flies over time. Median survival (50%) line shown. Median percent survival analyzed using the Mantel-Cox logrank test. Life span is significantly longer in R-R flies compared to R-C and R-H flies.   * $p < 0.05$. $n = 30$ flies per genotype.

was unexpected since in the R-C mutation is associated with the more severe seizure disorder Dravet syndrome, while R-H is associated with GEFS+.

Our initial observations suggested the mutant lines were more difficult to maintain over time than the control lines. Since seizure activity in humans can be associated with premature death and homozygous knock-in mice with the R-H mutation have reduced lifespan (Tang et. al. 2009, Martin et. al. 2010), this property was evaluated in the R-C, R-H and R-R lines maintained at 22ºC. Since individual lines from the same genotype displayed similar seizure phenotypes, one line from each genotype was chosen and used exclusively for the lifespan assay and the remainder of the studies. Both the heterozygous R-H and R-C flies display a markedly decreased median survival time of about 60 days when compared to R-R flies with a median survival of 73 days (Fig. 2C, p<0.05, Mantel-Cox logrank test). This similarity in severity between R-C and R-H was again unexpected because the R-H mutation is associated with the less severe GEFS+ seizure disorder.

Constitutive and heat-induced alterations in firing properties in R1648C and R1648H inhibitory neurons

Although behavioral studies facilitate an exploration of organismal dysfunction in the presence of R1648C and R1648H sodium channel mutations, studying neuronal firing and sodium current activity directly in the neurons of mutated flies is necessary to establish the underlying cellular mechanisms of dysfunction. In both mouse and fly knock-in models of *SCN1A*-epilepsy, alterations in sodium currents and excitability have been primarily observed in inhibitory neurons (Ogiwara et. al. 2007, Schutte et. al. 2016). Therefore, we focused on evaluating the effects of R-C and R-H mutations in inhibitory neurons. Though the

site of seizure generation in flies is unknown, local neurons (LNs) in the fly antennal lobes are a readily accessible population of GABAergic inhibitory neurons in the important and well-characterized olfactory circuit within the Drosophila brain (Seki et. al. 2010). To observe neuronal firing and sodium channel activity in these neurons, the whole brain was dissected from adult flies (Roemmich et. al. 2018), dorsal lateral local neurons were identified by their location and morphology, and whole-cell electrophysiological recordings were performed.

Evoked firing was evaluated by subjecting each cell to a series of depolarizing current steps, first at 22°C, following an increase in bathing solution temperature to 30°C, and finally after return of the bath solution to 22°C. Prior to heating, the majority of neurons in all three genotypes fired action potentials throughout the 600 ms current step (Fig. 3A-C). While a similar firing pattern persisted in the control LNs (Fig. 3Ai) at 30°C, heating resulted in sustained depolarizations and cessation of action potential firing in R-C and R-H neurons. In some cases depolarizations persisted after the current step (post-stimulus depolarization, Fig. 3Bi,Ci). Quantitative analysis of these events demonstrate that at 22°C, sustained depolarizations and post-stimulus depolarizations occurred in a minority of mutant neurons but were never seen in control neurons (Fig. 3D,E). At 30°C, the incidence of sustained depolarizations increased dramatically in the mutant neurons such that nearly all of R-C and R-H neurons exhibited these events (Fig. 3D). In contrast, there was no evidence of sustained depolarization or post-stimulus depolarization in the control R-R neurons even at elevated temperature. Post-stimulus sustained depolarization incidence increased as well, seen in the majority of mutant neurons at elevated temperature (Fig. 3E). Importantly, the reversal of the temperature induced changes in firing demonstrate that these alterations are associated

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with the mutation and not simply a result of irreversible damage to the cell induced by heating.
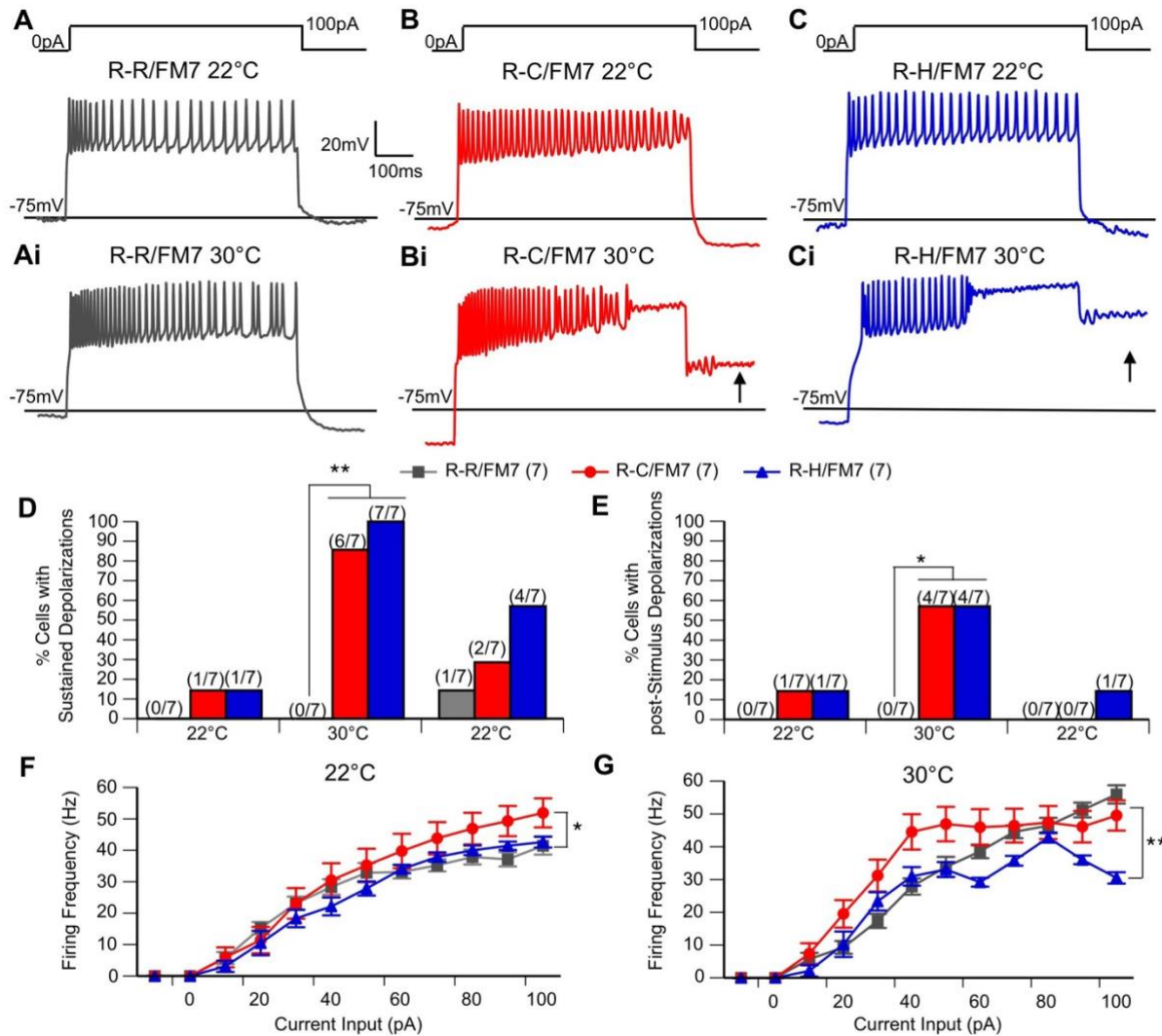
Figure 3. R-C and R-H mutants display sustained depolarizations during evoked firing, even after stimulus has ended. (A-C) Representative trains of action potentials evoked by illustrated stimulus protocol at 22˚C (A-C) and 30˚C (Ai-Ci). Arrow indicates appearance of post-stimulus depolarization. (D-E) % of cells in each genotype with sustained depolarizations during stimulus (F) and post-stimulus depolarizations (G) at 22˚C, at 30˚C, and after cooling back to 22˚C. R-C and R-H mutants have increased incidence of SDs and psSDs. *p<0.05, **p<0.01, Chi-squared. (F-G) Average spike frequency shown as a function of current input at ambient (F) and elevated (G) temperatures. At 22˚C, R-Cs have generally increased firing frequency. At 30˚C, R-Hs have generally decreased firing frequency compared to R-Cs. n = 7 groups per genotype. *p<0.05, **p<0.01. Two-Way ANOVA, Tukey's post-hoc test.

There were also differences in the firing frequency and response to temperature increase observed between control and mutant neurons. In control LNs the firing frequency increased with higher current inputs at both 22˚C and 30˚C (Fig. 3F,G). At 22˚C, R-C LNs displayed increased firing frequency compared to controls. However, for R-C and R-H LNs

the firing frequency increased with higher current inputs only at 22℃. At 30℃, particularly for R-H LNs, the firing frequency began to decrease at the larger current steps corresponding with the appearance of sustained depolarizations in which the firing of action potentials stopped but the cell remained depolarized, either for the remainder of the stimulus, or even after the current injection was over (post-stimulus sustained depolarizations) (Fig. 3F,G p<0.05, Two-way ANOVA).
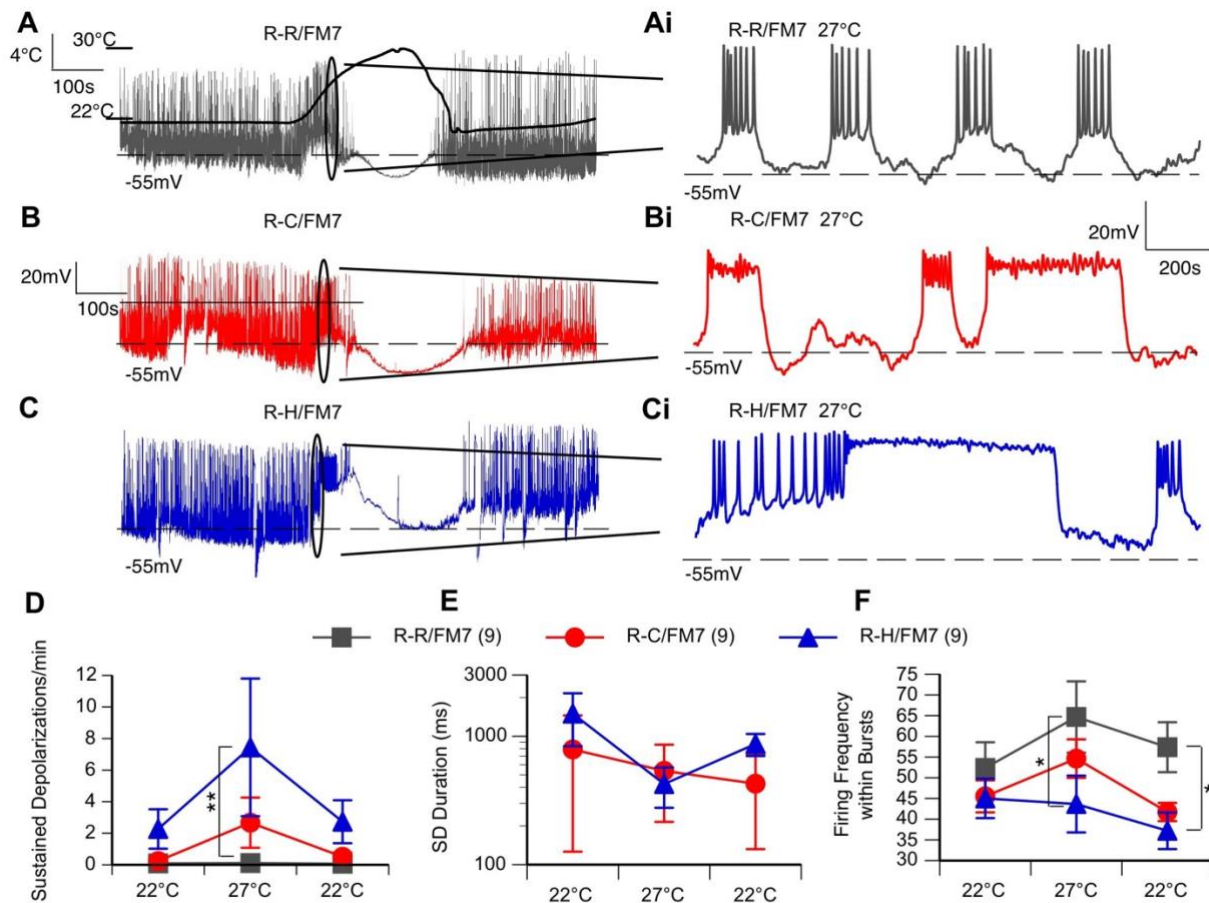
Figure 4. R-C and R-H mutants display spontaneous sustained depolarizations and reduced inhibitory firing frequency at elevated temperatures. (A-C) Representative continuous recording of neuron subjected to heating protocol. (Ai-Ci) 1.5s interval within the circled part of the temperature curve displaying sustained depolarizations. (D-E) Incidence and duration of sustained depolarizations. (F) Firing frequency of action potentials within bursts. n = 9 cells per genotype. * p<0.05, ** p<0.01. One-way ANOVA, Tukey's post-hoc test.

Although evoked firing frequency in mutant LNs is generally comparable to controls, the presence of sustained depolarizations in the mutants could cause overall decreased excitability in interneurons. Therefore we evaluated the spontaneous activity in LNs in the absence of holding hyperpolarizing or injected depolarizing current. To explore the effect of temperature, spontaneous neuronal activity in each cell was recorded continuously for 14 minutes: 5 minutes at 22°C, 4 minutes during heating to 30°C, and 5 minutes during cooling back down to 22°C. Both control and mutant neurons exhibited a regular burst firing pattern at 22°C that increased in frequency during heating before all firing stopped at 30°C. Action potential firing resumed following return to 22°C as illustrated in Figure 4 (A-C). Examination of the firing properties at an expanded time scale during the period of heating before cessation of activity illustrates the type of sustained depolarizations (>100 ms in duration) observed in both mutant lines but not in the control (Fig. 4Ai-Ci,D). Quantitative assessment reveals a significant increase in the incidence of sustained depolarizations in the R-H mutants as a function of elevated temperature that was not seen in the control neurons (Fig. 4D, $p<0.05$, Two-way ANOVA). The duration of the sustained depolarizations, averaging ~one second, was similar in the two mutants and the duration did not further increase with temperature (Fig. 4E). R-H neurons also displayed decreased firing frequency within bursts, particularly at elevated temperature (Fig. 4F, $p<0.05$, Two-way ANOVA). There were no significant differences between the mutants and control neurons in other aspects of spontaneous firing examined: resting potential, burst threshold, and burst amplitude (Table 1).

Table 1. *Properties of spontaneous burst firing in R-R, R-C and R-H LNs*

	Resting Potential, mV	Burst Threshold, mV	Burst Amplitude, mV
R-R/FM7	-57.13 +/- 2.21 (8)	-37.77 +/- 1.48 (8)	35.82 +/- 2.13 (9)
R-C/FM7	-54.21 +/- 1.96 (9)	-37.48 +/- 1.83 (9)	32.27 +/- 3.83 (9)
R-H/FM7	-53.98 +/- 2.59 (8)	-38.00 +/- 2.45 (8)	33.32 +/- 4.56 (9)

Values are means +/- SE at 22°C.

Alterations in sodium currents in R1648C and R1648H inhibitory neurons

To evaluate changes in the underlying sodium currents, voltage-clamp recordings were conducted on inhibitory neurons in R-R, R-C, and R-H flies at both ambient and elevated temperature. Sodium currents were activated by holding the cell below firing threshold at -75mV and applying a depolarizing voltage step in increasing 5mV steps. The sodium current waveform is similar in all three cells lines with an initial transient current (INaT) and a smaller persistent current (INaP) at both 22°C and 30°C (Fig. 5A). The significant delay between the stimulus onset and sodium current activation is due to the

recording electrode being located on the LN cell body which is electronically distant from the sodium channels located on the distal axonal segments in fly neurons (Ravenscroft et. al. 2020). This recording configuration prohibits adequate space-clamp preventing accurate

assessment of the time course of activation and inactivation. Additionally, the transient

sodium current amplitude in many neurons showed a decrease during the heating regime

(Fig. 5A-C) that did not recover when cooled (Fig. 5D). Therefore, we did not further

evaluate the potential effects of the mutation on peak current amplitude. In contrast, the

average activation threshold, defined as the least depolarized voltage step that activates the

first current, was stable throughout the recording. This property was significantly more

hyperpolarized in R-H compared to R-C and R-R but there was no effect of temperature (Fig.

5E, $p<0.05$, Two-Way ANOVA).
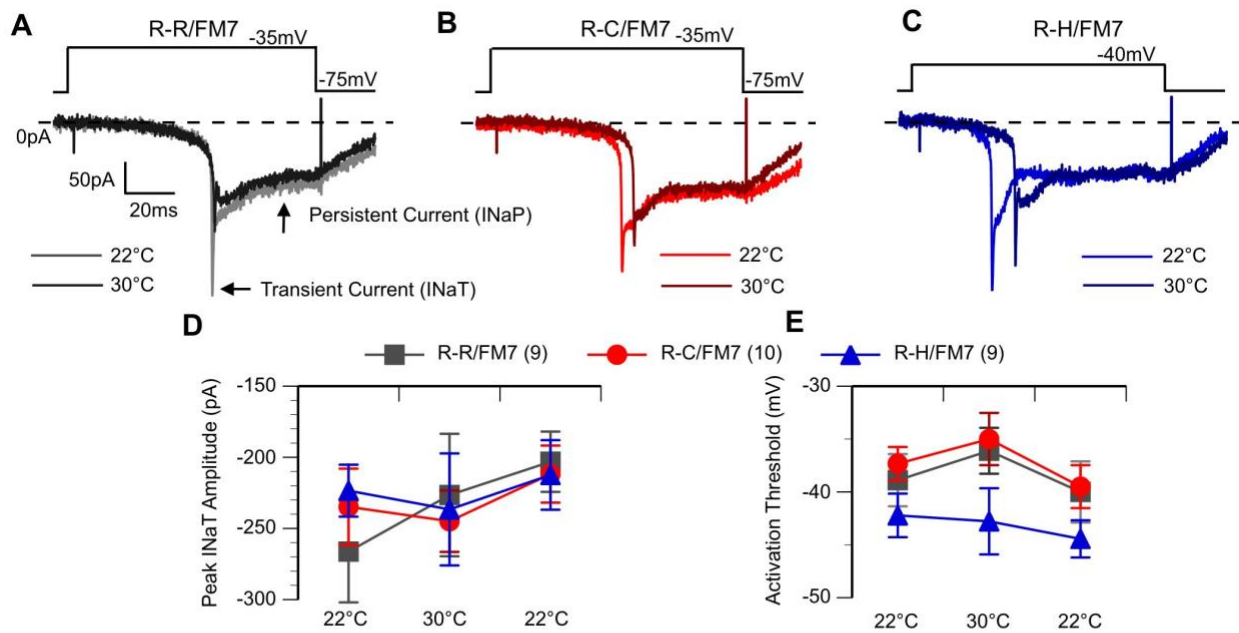
Figure 5. R-H neurons exhibit hyperpolarized sodium current activation threshold. (A-C) Voltage step-elicited currents at 22°C and h30°C. The first voltage-step to elicit current shown. Cells exhibit both an initial transient, and then persistent current. (D) Average peak INaT amplitude. (E) Average activation threshold at 22°C, 30°C, and following cooling (second 22°C). R-H mutants have an average activation threshold hyperpolarized to R-C and R-R, but no effect by temperature. * $p < 0.05$. Two-way ANOVA, Tukey's post-hoc test.

To examine the current deactivation properties, a multi-step protocol was employed.

The cell was first depolarized to a fixed voltage of -5 mV to activate sodium currents and then

returned to different resting potentials that varied in 10 mV steps between -95 and -25 mV

(Fig. 6A-C). Deactivation threshold was defined as the least negative voltage step resulting in current returning to baseline at the end of the stimulus. Both the R-C and R-H neurons had a significant hyperpolarizing shift in deactivation threshold (-55mV) compared to that of R-R controls (-40 mV) (Fig. 6D, $p<0.05$, Two-Way ANOVA). There was no effect of temperature on the deactivation. The deactivation data indicate that mutant sodium channels in inhibitory neurons are open over a wider voltage range, resulting in increased influx of sodium ions for the same stimulus in mutants compared to controls. However, unlike the abnormal firing properties, the sodium current alterations in deactivation were present at similar levels at both room and high temperature.
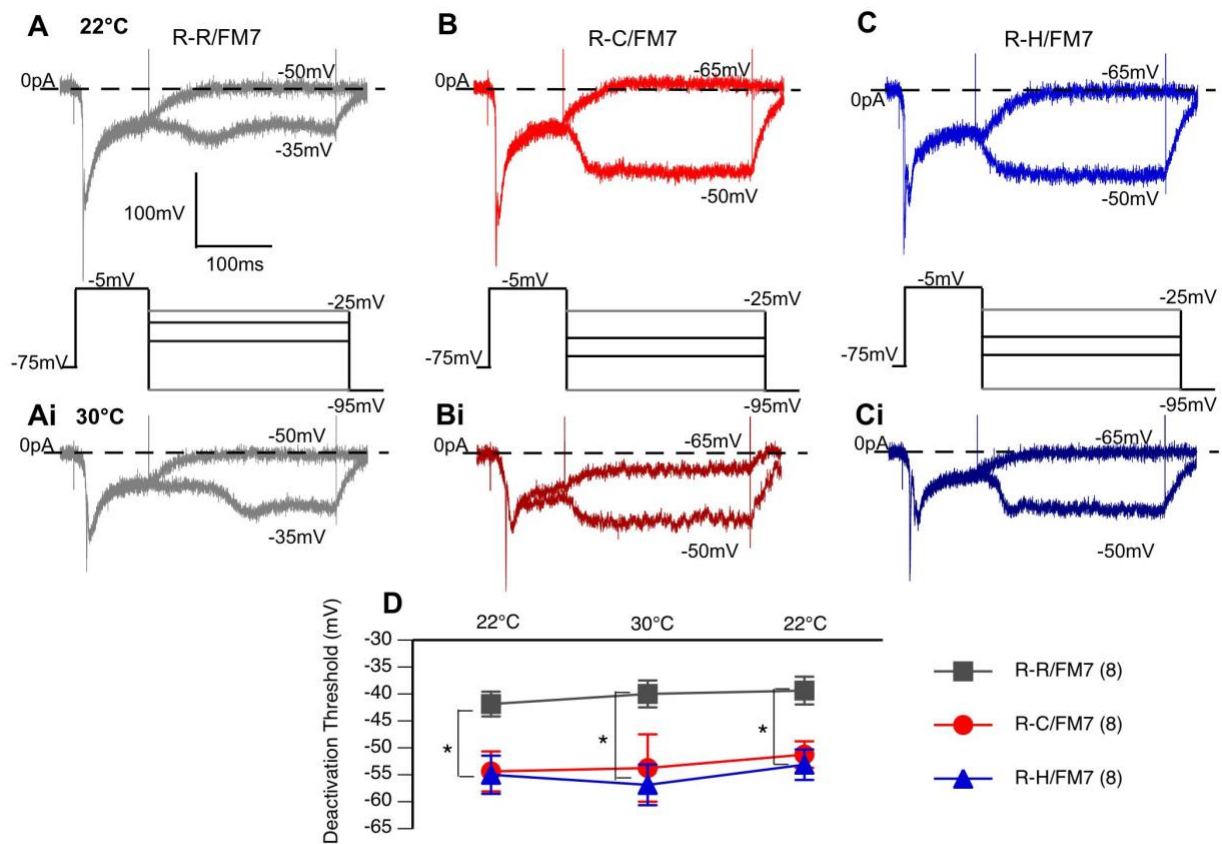
Figure 6. R-C and R-H neurons exhibit hyperpolarized sodium current deactivation threshold. (A-C) Voltage-step sodium channel currents for deactivation at room temperature (22°C) (A-C) and high temperature (30°C) (Ai-Ci), with the current steps in bold. Cells subjected to multi-step protocol as shown. Selected sweeps shown to illustrate differences in deactivation properties. (D) Average deactivation threshold at 22°C, 30°C, and after cooling back to 22°C. R-C and R-H mutants had hyperpolarized deactivation threshold compared to R-Rs. *p<0.05. Two-Way ANOVA, Tukey's post-hoc test.

3.4 Discussion

For patients with genetic epilepsy, the connection between a specific mutation and behavioral symptoms is largely unknown. Our study focused on *SCN1A*-mediated genetic epilepsies that arise from the same amino acid position at 1648. The R-C mutation is associated with the more severe disorder Dravet Syndrome, while the R-H mutation is associated with the milder disorder, GEFS+ (Baulac et. al. 1999, Ohmori et. al. 2002, Striano et. al. 2008, Escayg and Goldin 2010). This leads to the question of whether the differences in severity arise from distinct changes in channel function in the two mutants or if similar channel changes result in different behavioral outcomes depending on genetic background. Using independently generated Drosophila lines carrying either the R-H or R-C mutation we were able to compare the mutations' effects on cellular physiology and organismal behavior in a similar genetic background. We observed that both R1648C and R1648H mutations resulted in similar patterns and severity of cellular dysfunction, spontaneous and temperature sensitive seizure activity, and decreased lifespan. This suggests it is likely that genetic background contributes to difference in severity in patients with these mutations.

A role for genetic background in contributing to disease phenotype is consistent with clinical data indicating that the severity of seizure disorders can vary even between individuals with the same *SCN1A* mutation. For example, in a single multi-generational French family, R1648H was identified in thirteen individuals whose symptoms ranged from experiencing a few febrile seizures in early childhood to intractable epilepsy throughout adulthood associated with moderate or severe intellectual disability (Baulac *et. al.* 1999). This suggests interplay between the mutation and other factors are involved in determining

disease severity. This could include genetic background or environmental/developmental differences. Recent studies in human iPSC-derived neurons demonstrate that the GEFS+ causing K1270T mutation has differential effects on sodium currents and neuronal firing properties in different genetic backgrounds (Xie *et. al.* 2019). Transgenic Drosophila will be valuable in assessing the interaction between specific epilepsy causing missense mutations and genetic background in manifestation not only of cellular dysfunction but also in behavioral phenotypes.

Previous studies using mice have been important in understanding cellular mechanisms associated with several epilepsy causing sodium channel mutations, including a knock-in model of the missense mutation R1648H (Tang et. al. 2009, Martin et. al. 2010). While the phylogenetic distance between humans and flies is greater than mice, that are some distinct advantages to conducting parallel studies in Drosophila. The DNA sequences that encode voltage-gated sodium channels are highly conserved between flies and mammals, but Drosophila has only one voltage-gated sodium channel gene (para) (O'Dowd et. al. 1989, Loughney et. al. 1989). In contrast, there are nine different voltage-gated sodium channel alpha subunit isoforms in mice and other mammals, three of which are highly expressed in the nervous system (*SCN1A, SCN2A, SCN8A*) (Catterall 2017). These isoforms can and do interact in models of patient epilepsies. For example, mice that were double heterozygous mutants for *SCN1A* and *SCN8A* had restored seizure susceptibility and lifespan compared to mice heterozygous for only *SCN1A* (Martin et. al. 2007). In addition, creation and maintenance of multiple different mouse lines carrying distinct missense mutations in different genetic backgrounds is a time and resource intensive proposition. In the present study, the transgenic flies used were created using a two-step CRISPR/Cas9 gene editing

method that involved introducing a visible marker in the first step. This resulted in efficient detection of multiple founder flies with the desired mutations in a similar genetic background. In addition, the low cost of maintaining multiple lines, including control substitutions in addition to the mutants, was an attractive reason to use Drosophila.

Analysis of two previous Drosophila knock-in models of *SCN1A* missense mutations, one causing Dravet Syndrome (S1231R) and one causing GEFS+ (K1270T) exhibited alterations in sodium current and firing properties only in animals homozygous for the mutations (Schutte et. al. 2016). Heterozygotes exhibited moderate seizure behavior at high temperatures but no obvious alterations in neuronal activity (Sun et. al. 2012, Schutte et. al. 2014). Therefore, the finding that fly lines homozygous for the R-C or R-H mutations were lethal, and that heterozygotes displayed significant seizure activity and reduced lifespan, was somewhat unexpected. The location of the 1648 mutation on the voltage-sensing region of *SCN1A* particularly crucial for inactivation may explain this increased level of severity compared to the previously examined DS (S1231R) and GEFS+ (K1270T) mutations. The R-C mutation substitutes a neutral amino acid for a positive gating charge. R-H conserves charge, but adds steric hindrance. Interestingly, in a study investigating the effects of an R-H mutation in the S4 segment of domain IV of human skeletal sodium channel expressed in HEK cells, changing the pH to protonate the histidine did not change the functional alterations of the mutation (mixed inactivation defects) (Alekov et. al. 2000). This suggests that the steric effects of the histidine side-chain may be the primary molecular culprit behind phenotype associated with the R1648H mutation. Changing the size and/or charge of one of the gating charges on the S4 segment in domain IV likely effects its ability to exchange ion binding partners with the environment. Given our observations that R1648H or R1648C

mutations in inhibitory neurons result in sustained depolarizations and require repolarization to more negative voltages to deactivate the sodium current, both mutations likely impact the ability of the S4 segment in domain IV to move back into place, closing the channel (Ahern et. al. 2016). The S4 segment in domain IV of *SCN1A* may therefore play a previously undescribed role in deactivation, as well as inactivation.

The R-C and R-H inhibitory neurons exhibited heat-induced alterations in neuronal firing properties including appearance of sustained depolarizations, post-stimulus sustained depolarizations, and decreased firing frequency during both evoked and spontaneous activity. The temperature dependent aspect of these changes suggests they are likely to contribute to the heat-induced increases in seizure activity. Analysis of the underlying sodium currents in mutant neurons revealed a hyperpolarized deactivation threshold in R-C and R-H neurons even at room temperature. This is consistent with the R-H and R-C sodium channels being more difficult to close once activated which would result in increased ion flow, either due to longer channel openings, and/or channel opening to a greater extent. Dravet Syndrome has most often been attributed to loss-of-function mutations, as was the case in the previous S1231R Dravet Syndrome knock-in model in Drosophila. The current result indicates that gain-of-function can also contribute to Dravet. However, unlike the abnormal firing properties, mutant sodium current alterations were present at both room and high temperature. This suggests the increased inward flow of sodium ions is not sufficient to trigger the sustained depolarizations and reduced firing frequency seen at high temperature by themselves but through an interaction with a different and as yet unidentified heat-induced change.

One heat-induced aspect of cellular physiology that could be involved are calcium currents. Previous studies have found that calcium currents exhibit increased conductance at elevated temperature in rat brain slices (Radziki et. al. 2013). Previous studies have shown that calcium currents also contribute to membrane depolarization in Drosophila neurons, including in the neurons located in the adult fly brain (Peng and Wu 2007, Gu et. al. 2009). In projection neurons in the adult antennal lobes, the calcium currents have both a transient and sustained component. The *alpha1T* gene encodes a CaV3-type calcium channel mediating the transient current (Iniguez et. al. 2013) and the *cac* gene encodes a Cav2-type calcium channel mediating the sustained current (Gu et. al. 2009). If similar calcium currents are present in antennal lobe LNs, it is possible that potassium currents are sufficient in most cases for repolarizing the action potential, even with the increased sodium conductance in the mutants due to hyperpolarized deactivation of the channels at room temperature, resulting only rarely in sustained depolarizations. However, at high temperature an increased magnitude of calcium currents, in conjunction with increased magnitude of the sodium currents in the mutants, could more severely impede the ability of potassium currents to repolarize effectively, resulting in more sustained depolarizations. Future studies, taking advantage of both forward and reverse genetic approaches that are relatively easy in Drosophila, will be important in identifying the interacting variable/s associated with the heat-induced change in firing properties.

In the mouse model, the primary effect of the R1648H mutation is on inhibitory neurons where a decrease in inhibitory neuron activity can lead to unbalanced over-excitability in the brain and seizures (Martin et. al. 2010). The present study focused exclusively on LNs, inhibitory neurons in the antennal lobe that processes olfactory

information in Drosophila. While the para sodium channel in Drosophila undergoes extensive alternative splicing (Loughney et. al. 1989, Lin et. al. 2009), the R1648 amino acid is located in an obligate exon so mutations at this site will be in present in all neurons (Lin et. al. 2009, Ravenscroft et. al. 2020). Therefore, it will be interesting in future studies to also evaluate the effect of this mutation on excitatory neurons in the same antennal lobe circuit.

Taken together, the results of the present study demonstrate that R1648C and R1648H *SCN1A* mutations result in a gain-of-function in inhibitory neuron sodium channels in Drosophila. The incomplete closing and/or extended opening of the sodium channels could cause an inappropriate influx of sodium ions which in turn causes impaired inhibitory neuron firing. Neuronal network activity is based on a balance between inhibitory and excitatory activity, and with the impaired firing of inhibitory neurons, the balance is tilted in favor of overexcitation, which can lead to seizures. CRISPR-generated *Drosophila* lines represent low-cost platforms that can be used to elucidate neuronal mechanisms of seizure generation and guide development of new patient-specific therapies for epilepsy. Patient-specific studies using model organisms, including Drosophila, will be an important tool in our quest to improve our mechanistic understanding of genetic epilepsy and develop better patient-specific treatments.

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CHAPTER IV

Cell-type Specificity of Firing Properties Caused by R1648C/H Mutations

4.1 Introduction

In mammals, the Na$_V$1.1 voltage-gated sodium channel is largely localized to neurons in the CNS, and has been found at particularly high density at the axon initial segments of parvalbumin-positive inhibitory interneurons (Ogiwara et. al. 2007). This has led to the suggestion that Na$_V$1.1 may play a particularly important role in modulating excitability in neural networks. In the first mouse model of a human SCN1A epilepsy syndrome, it was reported that sodium current magnitude and evoked firing were reduced in inhibitory hippocampal interneurons but not in excitatory pyramidal neurons (Yu *et. al.* 2006.) Two subsequent studies using a knock-in mouse model of GEFS+ associated with an R1648H mutation, also demonstrated that sodium current amplitude and firing properties were selectively reduced in bipolar inhibitory neurons (Tang et. al. 2009, Martin et. al. 2010). Furthermore, Na$_V$1.1 haploinsufficiency in inhibitory neurons alone has led to epileptic seizures and premature death in a Dravet Syndrome mouse model (Cheah et. al. 2012, Ogiwara et. al. 2013), while insufficiency in excitatory neurons alone did not (Ogiwara et. al. 2013). Additionally, in a recent model of heterozygous R1648H mice, inhibitory hippocampal neurons had depolarized action potential thresholds, increased halfwidths, and decreased amplitudes, while excitatory hippocampal neurons displayed no differences to control neurons (Das et. al. 2021). While alterations have occasionally been found in both inhibitory and excitatory neurons in several models of genetic epilepsy (Liu et. al. 2013, Xie et. al. 2019, Schutte et. al. 2016), the cell-type specific effects of these alterations differ between models. In an IPSC model of K1270T in SCN1A, there were decreases in sodium current, firing frequency and amplitude in inhibitory neurons, but also a decrease in sodium current in excitatory neurons. However, this decrease in sodium current in excitatory

neurons was not sufficient to reduce excitability in these cells (Xie et. al. 2019). These studies strongly support the hypothesis that epilepsy caused by mutations in SCN1A are due to reduced sodium currents and excitability, primarily in inhibitory neurons.

The question of cell specificity has also been explored in two different Drosophila models of SCN1A epilepsy. In Drosophila, the voltage-gated sodium channel Para is expressed broadly in the adult CNS, and located at axonal segments distal to the site of dendritic integration (Ravenscroft et. al. 2020). In a knock-in model of Dravet Syndrome caused by the S1231R SCN1A mutation, inhibitory cells had decreased firing with no detectable effect on excitatory neurons (Schutte et. al. 2014, Schutte et. al. 2016). In a model of GEFS+ with the K1270T SCN1A mutation, inhibitory cells had decreased firing and excitatory neurons displayed increased firing frequency (Sun et. al. 2012, Schutte et. al. 2016.) In both cases the GABAergic local neurons (LNs) were in the antennae lobe but the excitatory neurons examined were glutamatergic motor neurons located in the adult thoracic ganglion. Thus, while inhibitory neurons appear to be more affected than excitatory neurons in the Drosophila models as well, the two populations of cells studied were not in the same neural circuit.

In our study the goal was to determine if R1648 mutations have a similar cell-type specificity of action in the Drosophila model as in the mouse and human iPSC models. Therefore, we examined evoked firing properties of excitatory projection neurons (PNs) located in the antennal lobe. In the antennal lobe, olfactory receptor neurons terminate onto cholinergic PNs that project to higher processing areas including the mushroom bodies and lateral horn (Vosshall and Stocker 2007). The inhibitory local neurons previously studied in

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this thesis project diffusely on the antennae lobes to regulate circuit excitability (Wilson et. al. 2013). The excitatory projection neurons are cholinergic, rather than glutamatergic, but they are in the same circuit as the previously described inhibitory neurons, providing the ability to compare inhibitory vs excitatory balance in the same network.

4.2 Methods

Adult fly brains were dissected as previously described. Briefly, the fly was anesthetized on ice, decapitated, and the whole brain removed from the head capsule in an external solution containing papain (Roemmich et. al. 2018, Roemmich et. al. 2021). For recording from PNs, the most medial group of cells in each antennae lobe were targeted, a locus of cells distinct from the more lateral cluster of LNs previously studied. PNs were further identified by their firing properties. Evoked firing was assessed at ambient (22℃) and elevated (30℃) temperatures.

4.3 Results

To determine if the PNs in R-C and R-H mutants exhibited similar changes in firing properties to those observed in LNs, whole cell recordings were obtained from PNs with a resting membrane potential of <= -30 mV upon break in. Neurons were held at a membrane potential of -75 mV for the duration of the recording. At room temperature, depolarizing current steps elicited trains of small amplitude spikelets in PNs in all three genotypes (Fig. 1A-C). The spikelets represent action potentials initiated at sites electrotonically, typical of PNs (Schutte et. al. 2016). Trains of spikelets persisted throughout the depolarizing step when the temperature was elevated in all three genotypes (Fig. 1Ai-Ci). In addition, there was no appearance of a post-stimulus depolarization in PNs from either mutant (Fig. 1D).

This is distinct from the behavior of LNs from the mutants which showed discontinuous firing during a sustained stimulus and post-stimulus depolarizations, particularly at elevated temperature.

To assess the firing frequency as a function of current step, and the effect of temperature, we counted each spikelet defined by a voltage change that included an inflection point on the rising phase and was a minimal size of 1 mv. The spikelet amplitude decreased at elevated temperature, and for many cells a decrease in signal to noise ratio
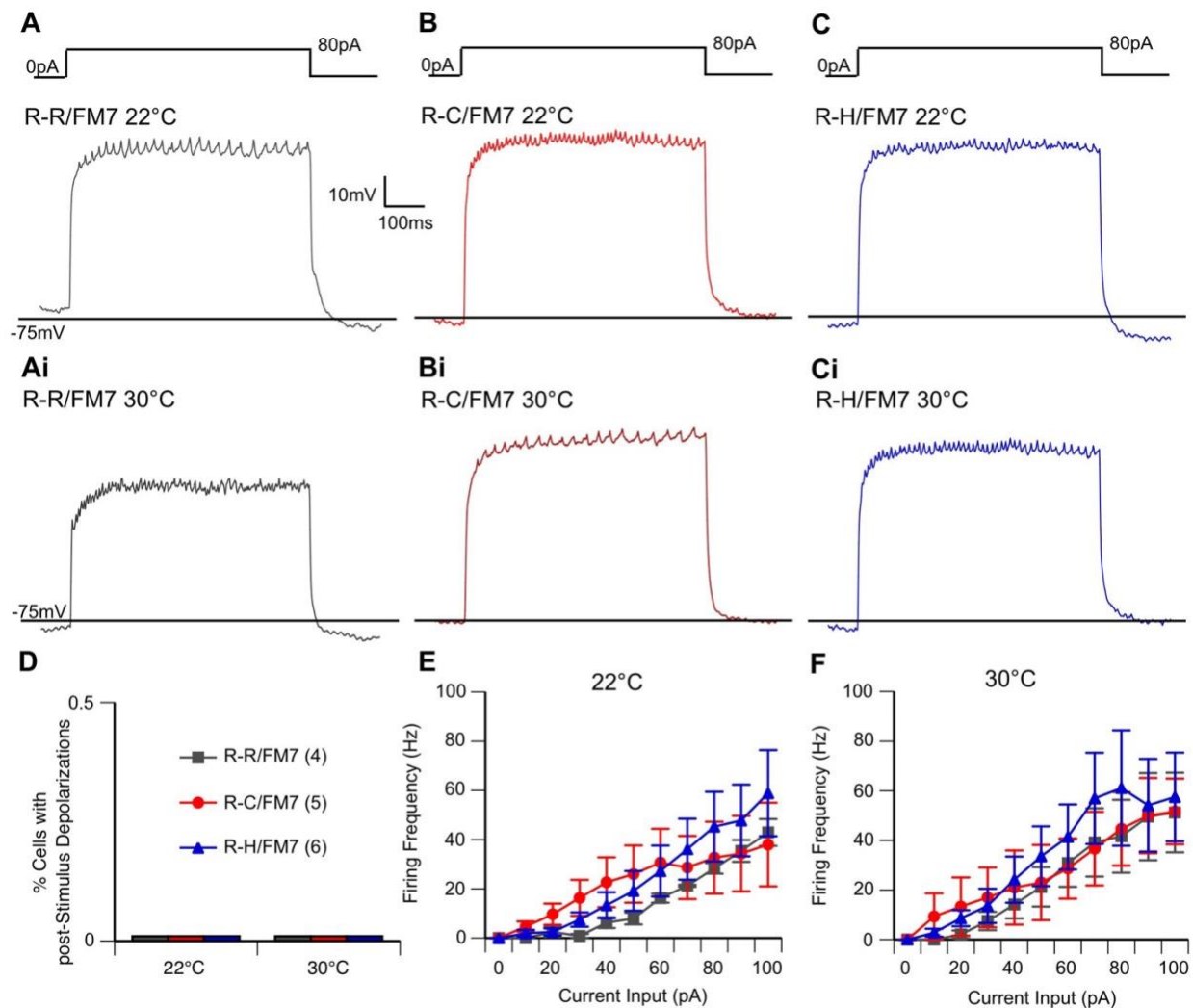
Figure 1. R-C and R-H projection neurons do not display post-stimulus sustained depolarizations. (A-C) Representative trains of action potentials evoked by illustrated stimulus protocol at 22°C (A-C) and 30°C (Ai-Ci). (D) % of cells in each genotype with sustained depolarizations during post-stimulus depolarizations at 22°C, at 30°C. R-C and R-H PNs have no incidence of psSDs, in contrast to mutant LNs. (E-F) Average spike frequency shown as a function of current input at ambient (E) and elevated (F) temperatures. At 30°C, R-Hs have generally increased firing frequency compared to R-Rs. *p<0.05, Two-Way ANOVA, Tukey's post-hoc test.

precluded this analysis (Fig. 1Ai-Ci). For PNs in which it was possible to distinguish the spikelets from noise at the high temperature the data suggest similar patterns of firing in R-C and R-H PNs compared to R-R (Fig. 1E-F, two-way ANOVA). The similarity between genotypes in PN firing differs from findings in LNs, in which R-C LNs displayed a small but significant increase in evoked firing at room temperature compared to controls, and R-H LNs displayed a significant decrease in evoked firing at 30°C compared to controls. Similar to the LNs, there were no significant different in the passive membrane properties in PNs between the genotypes (Table 1).

Table 1. *Membrane properties of R-R, R-C and R-H PNs*

	Input Resistance (GΩ)	Cell Capacitance, pF	RMP, mV
R-R/FM7	0.62 +/- 0.06 (8)	5.80 +/- 0.60 (4)	-52.35 +/- 4.47 (4)
R-C/FM7	0.61 +/- 0.11 (5)	5.92 +/- 1.04 (5)	-51.12 +/- 6.83 (5)
R-H/FM7	0.61 +/- 0.07 (8)	6.36 +/- 3.23 (5)	-51.86 +/- 11.66 (6)

Values are means +/- SE at 22°C.

4.4 Discussion

These data indicate that SCN1A mutations at the R1648 location alter firing properties in a cell-type specific fashion in the Drosophila model. Similar to studies in the mouse knock-in model (Martin et. al. 2010), the R-H mutation caused a reduction in firing of

inhibitory interneurons in Drosophila but it did not reduce the firing of excitatory neurons. While the R-C mutation has not been studied in a mouse knock-in model, the R-C mutation in Drosophila caused a similar reduction in firing of inhibitory interneurons without apparent reduction in firing of excitatory neurons. The consistent findings between two distantly related model systems suggest that the cell type-specific reduction in firing is likely to be an important feature of the disease phenotype in human patients.

Analysis of the firing frequency in PNs was complicated by the small amplitude of the spikelets that decrease with increasing temperature, and the heterogeneity of evoked firing responses within PNs of the same genotype. However, recordings of evoked firing at room and ambient temperatures failed to demonstrate a clear change in firing frequency between mutants and controls. Additionally, sustained depolarizations, particularly post-stimulus sustained depolarizations, were not seen in any PNs, starkly contrasting the majority of the R-C and R-H LNs that displayed post-stimulus sustained depolarizations at elevated temperature. These findings share similarities to both previous Drosophila knock-in models of SCN1A epilepsy, K1270T and S1231R, but do not repeat either models' findings.

In K1270T in Drosophila, an increase in the firing frequency of excitatory neurons was observed in concert with a decrease in firing of inhibitory neurons, and sustained depolarizations in inhibitory neurons were not seen in excitatory neurons. Though R1648H and R1648C local neurons shared many cellular phenotypes to K1270T LNs, particularly decreased inhibitory firing and sustained depolarizations excluded from PNs, R1648 LNs did not show the corresponding increase in excitatory PN firing (Sun et. al. 2012, Schutte et. al. 2016). In fact, our preliminary studies on PNs in R1648H and −C flies found no apparent

differences between mutants and controls. This is similar to PNs from S1231R Drosophila, though the findings in R1648H and –C LNs much more closely resemble the previous K1270T flies than the S1231R Drosophila (Schutte et. al. 2014, Schutte et. al. 2016). In K1270T iPSC-derived neurons, a reduced sodium current density in excitatory neurons was not sufficient to reduce excitatory firing, though a reduction in sodium current density in inhibitory neurons did contribute to reduced inhibitory firing (Xie et. al. 2020). In R1648C and R1648H flies, a reduction in inhibition without a corresponding reduction in excitation would tip the balance of the network to overexcitability and seizure susceptibility. Findings from the similar fly and iPSC models also continue to suggest that inhibitory neurons seem to be most impacted by SCN1a mutations, and also that there is diversity in the possible relationships between inhibitory and excitatory cellular phenotypes within a model.

The PNs in this study were located in the medial lateral antennal lobe cluster. While this cluster also contains some LNs, (Wilson et. al. 2013) we think it unlikely that we were recording from LN since the two cell types can be distinguished by their distinct firing phenotypes. PNs, identified in previous studies by intracellular labeling with biocytin or using a fluorescent marker driven by GAL4, with an axonal projection from the antennal lobe to the mushroom bodies, have very small spikelets, 1-4 mV. In contrast the LNs have axonal/dendritic projections confined to the antennal lobe and much larger action potentials (10-40 mV) (Chou et. al. 2010, Seki et. al. 2010).

In mammals there are three different sodium channel genes expressed in the adult nervous system (Catterall 2010). Cell type specific changes in activity associated with mutations in a single sodium channel gene could be attributed to differential expression of

these channels in different cell types, or differential regulation of other sodium channel genes in a compensatory fashion. There is only a single sodium channel gene in Drosophila (para) but this gene undergoes extensive alternative splicing (Lin et. al. 2009). Since the R1648 residue is located in an obligate exon, the R-H and R-C mutations should be present in all neurons. Therefore it seems most likely that projection neurons are less affected than local neurons due to different overall ion channel makeup, with voltage-gated sodium channels serving as a smaller proportion of ion channels in projection neurons. Even if inhibitory neurons are the only cell type affected by a mutation, a decrease in inhibitory neuron activity can lead to unbalanced over-excitability in the brain and seizures. Knowing which neuronal subtype(s) are the most impacted in patient-specific mutations, and how, can help with patient-targeted care.

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CHAPTER V

SCN1A R1648C and R1648H Drosophila are Hypoactive During the Day

The work in this chapter was conducted in collaboration with Dr. Ceazar Nave, Dr. Lisa Baik, and Dr. Todd C. Holmes at University of California – Irvine. Dr. Nave also ran the ClockLab sleep analysis.

5.1 Introduction

Patients with epilepsy often display sleep disorders. This is not surprising as seizures can impact large areas of the brain, including the hippocampus, frontal cortex, and thalamus (Papale et. al. 2013). Additionally, seizures during the day can lead to a decrease in REM sleep the following night, and seizures occurring during sleep decrease sleep quality even more, leading to tiredness the next day (Bazil and Walczak 1997). Furthermore, a decrease in sleep has resulted in more seizures (Pratt et. al. 1968, Badawy et. al. 2002), though it is controversial whether this is due to decreased sleep alone, or in conjunction with external factors such as stress (Malow et. al. 2002). In either case, sleep disruption and epilepsy disorders are closely related, and SCN1A-mediated epilepsies are no exception.

In one of the major SCN1A-mediated epilepsies, Dravet Syndrome, the vast majority of children (75-97%) have difficulty falling and staying asleep according to parental reports (Nolan et. al. 2006, Villas et. al. 2017, Licheni et. al. 2018). *Scn1a* transcripts are expressed broadly in cell types involved in both epilepsy and the sleep-wake cycle, including the hippocampus and cortex (Papale et. al. 2013). SCN1A knock-out mice (Dravet Syndrome) exhibited lower sleep quality and increased incidence of brief wakes during sleep but similar sleep duration to control mice in EEG-EMG studies. This contributed to decreased sodium current in GABAergic neurons of the reticular nucleus of the thalamus, the activity of which oscillates with excitatory neurons in the ventrobasal nucleus of the thalamus to regulate sleep (Kalume et. al. 2015).

The association of sleep disorders specifically with GEFS+ has been much less documented, though a study of R1648H knock-in mice showed increased wakefulness over a 48-hour diurnal cycle, including reduced REM sleep (Papale et. al. 2013). A sleep study in

an SCN1A-mediated GEFS+ mutation has also been studied in Drosophila. Drosophila have been a key model organism in the study of circadian rhythms and sleep (Zehring et. al. 1984, Vosshall et. al. 1994, Hendricks et. al. 2000, Shaw et. al. 2000, among many others). Sleep in flies is defined as five minutes or more of inactivity, and shares many characteristics of mammalian sleep, such that it is controlled by circadian and homeostatic regulation; associated with increased arousal thresholds, altered brain activity and posture; responsive to hypnotic and stimulant drugs, and controlled by highly conserved pathways. Flies with the GEFS+-causing SCN1A mutation K1270T surprisingly had decreased nighttime activity coinciding with increased sleep, but also greater daytime activity, contraindicating a locomotor deficit (Petrucelli 2015). These flies were previously shown to have hyperactive activation and deactivation sodium current thresholds in inhibitory neurons resulting in sodium currents active over a greater voltage range (Sun et. al. 2012). The sleep study on these flies suggested that their enhanced GABAergic transmission inhibited pigment dispersing factor (PDF) clock neurons that are wake-promoting, and that light played an important role in the sleep phenotype (Petrucelli 2015). Besides the inherent value of studying the circadian rhythms and sleep in flies to better understand these biological processes, studying circadian rhythms and sleep in Drosophila can be an important tool to assess cellular mechanisms of disease.

5.2 Methods

Locomotor activity of individual flies was recorded using the TriKinetics Drosophila Activity Monitoring System via infrared beam-crosses in 1-minute bins and analyzed in 1-minute bins (for sleep analysis) or 30-minute bins (for activity analysis) for seven days of 12-hour light/12-hour dark followed by five days of 24-hour darkness. Flies that died

during the trial were excluded from all activity analysis. By convention, the time of lights-on was denoted as ZT0 (Zeitgeber time) and the time of lights-off as ZT12. In accordance with the natural environment, lights were turned on at 06:30 h (ZT0) and off at 18:30 h (ZT12). The first two days of data were excluded to allow flies to adjust to the environment. Statistics were measured using FaasX and ClockLab software for activity and sleep analysis, respectively.

5.3 Results

Mutants are hypoactive during the daytime in light/dark conditions

To see if R1648C and R1648H flies displayed common epilepsy-associated behaviors other than seizure-like activity, we evaluated activity and sleep as circadian-modulated outputs for R-C, R-H, and R-R mutant flies. Flies housed individually in Drosophila Activity Monitors were exposed to a diurnal light/dark (LD) schedule (12hr lights on: 12hr lights off) for five days, followed by constant darkness (DD) for five days. Activity levels were assessed as a measure of infrared beam breaks by flies in the tubes. In a standard 12L:12D schedule, R-R controls exhibit morning and activity peaks in anticipation of light/dark or dark/light transitions, with a siesta during the day (Fig. 1A). R-H and R-C mutants exhibit significantly decreased activity levels during the day in LD, particularly during morning and evening activity peaks (Fig. 1A, $p<0.05$, Two-way ANOVA). Average daily activity count is also significantly decreased during daytime hours in LD (Fig. 1B, $p<0.01$, Two-way ANOVA).

Sleep in Drosophila is defined as five minutes or more of inactivity. Corresponding to decreased activity during portions of day for R1648C and R1648H mutants, sleep analysis
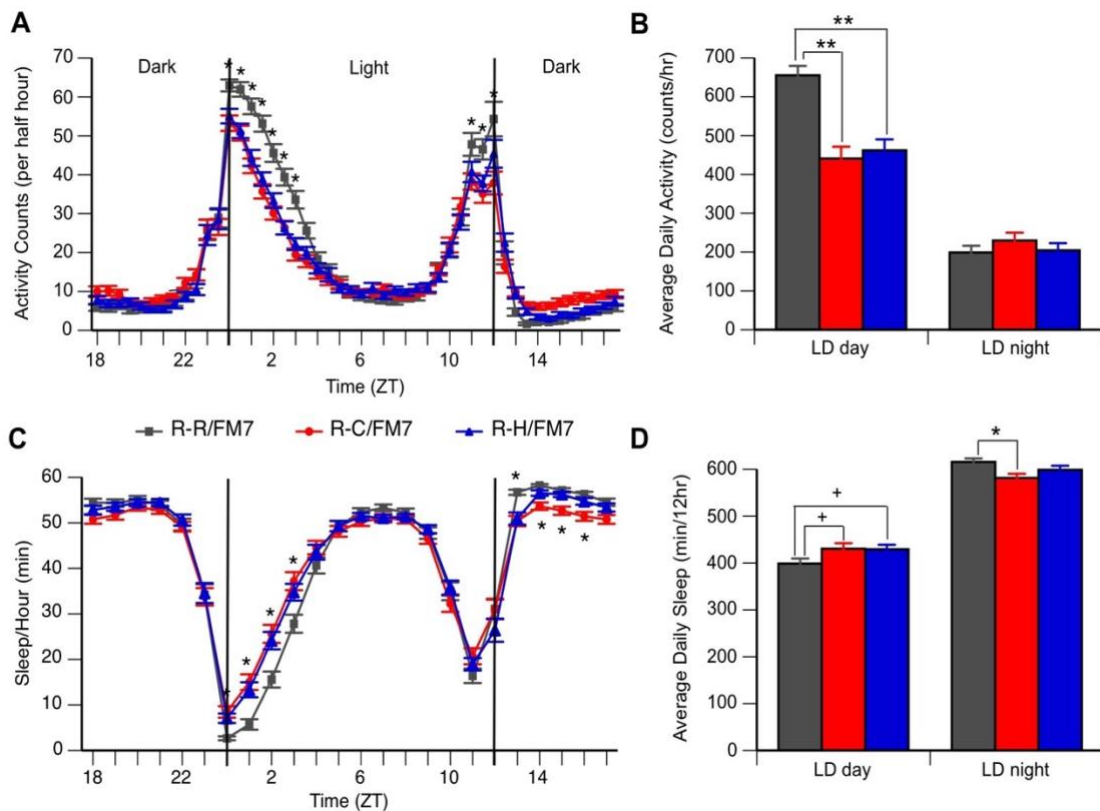
Figure 1. **Decreased daytime activity during LD in mutants.** (A) Actogram displaying average daily pattern of activity in light/dark conditions. (B) Overall average daily activity count totals during the day (ZT 0-12) and night (ZT 12-24) in light/dark conditions. (C) Average minutes of sleep per hour in light/dark conditions. (D) Overall average daily sleep min totals during the day (ZT 0-12) and night (ZT 12-24) in light/dark conditions. n = 79-81 animals for each group. * p < 0.05 between R-Rs and mutants. + 0.1 > p > 0.05. Two-way ANOVA, Tukey's post-hoc test.

indicates that both R1648C and R1648H flies have increased sleep during some daytime hours. R1648C mutants also have decreased sleep during nighttime hours (Fig. 1C, p<0.05, Two-way ANOVA). Both R-C and R-H mutants have average daily daytime sleep totals that trend higher than controls (Fig. 1D, p = 0.54, 0.65, Two-way ANOVA), and R-C mutants show significantly lower total nighttime sleep in LD (Fig. 1D, p<0.05, Two-way ANOVA). These data suggest that R-C and R-H flies tend to sleep inappropriately during daytime hours in light/dark conditions.

Mutants are hypoactive during the daytime in dark/dark conditions

To assess free running circadian activity in the absence of light cues, five days of constant darkness followed the week of light/darkness. Flies gradually transition their behavior in response to constant darkness, thus activity and sleep levels are shown at day 2 and day 5 of DD (Fig. 2A-F). Decreased activity levels during daytime hours in mutants persists into the transition into complete darkness (Fig. 2C, $p < 0.05$, Two-way ANOVA). Sleep levels after two days in constant darkness are similar between mutants and controls (Fig. 2D). However, by DD day 5, R-H mutants display increased levels of sleep during both daytime and nighttime hours (Fig. 2E,F, $p < 0.05$, Two-way ANOVA).
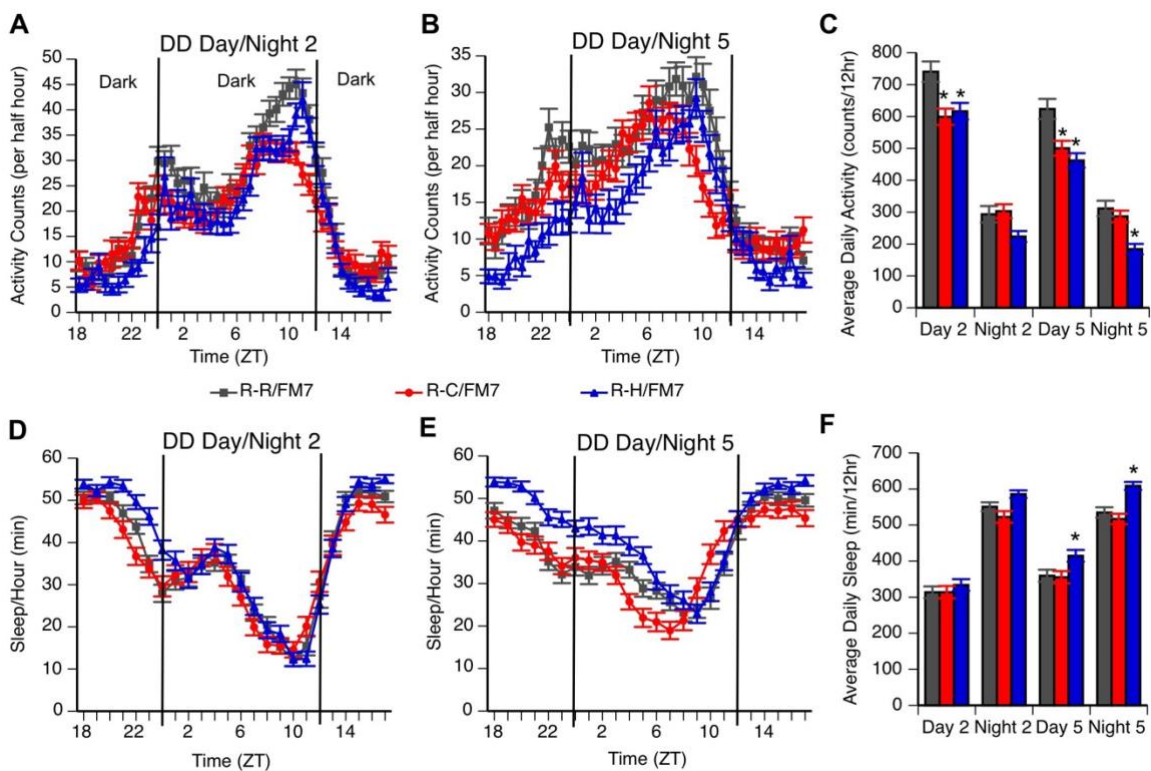
Figure 2. **Decreased daytime activity during DD in mutants.** (A-B) Actogram displaying average daily pattern of activity in light/dark conditions during day/night 2 (A) and day/night 5 (B) of DD. (C) Overall average daily activity count totals during the day (ZT 0-12) and night (ZT 12-24) in light/dark conditions. (D,E) Average minutes of sleep per hour in light/dark conditions during day/night 2 (D) and day/night 5 (E) of DD. (F) Overall average daily sleep min totals during the day (ZT 0-12) and night (ZT 12-24) in light/dark conditions. n = 79-81 animals for each group. * $p < 0.05$ between R-Rs and mutants. + $0.1 > p > 0.05$. Two-way ANOVA, Tukey's post-hoc test.

5.4 Discussion

Taken together, R-C and R-H mutant flies display interrupted bouts of activity and sleep duration. Daytime activity levels are greatly decreased in both R-C and R-H mutants, and seem to be more impacted than sleep levels, though the two measures are correlated. Seizure, lifespan, and altered activity seen in our mutant flies are features that reflect phenotypes that are typically observed in human patients. Further studies are recommended to explore the circadian activity and mechanisms of these mutants in depth.

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CHAPTER VI

Going Back to Show and Tell: Using Graduate Teaching Assistant Research to Engage Undergraduates

This work is in submission as Roemmich AJ Mauzy-Melitz D, Shaffer (2021) *CourseSource*

6.1 Introduction

At universities around the country and around the world, teaching assistants are responsible for leading STEM-discipline discussion sections, lab courses, and being the link between undergraduate students and faculty instructors of record. Often, these teaching assistants are graduate students conducting research of their own, who may not be familiar with or comfortable with teaching. Foundational studies examining the effectiveness of graduate teaching assistants (GTAs) found that GTAs were concerned with establishing credibility (1) and engaging their students (2).

More recent studies on the advantages and disadvantages of GTAs as seen by undergraduates report that GTAs' specified knowledge is often seen as problematic, but can be thought of as beneficial for some, "[bringing] the latest perspective or research in that field" (3). Additionally, while one study found that undergraduates perceive GTAs as hesitant, nervous, and uncertain, they were also seen as interactive, engaging, and relatable – an advantage GTAs have over professors, who are perceived as more organized, experienced, and confident than GTAs, but also distant, boring, and out-of-touch (4).

How can GTAs, especially those that are novice or unsure, gain confidence in a discussion section while capitalizing on their advantages of being up-to-date in the field and more relatable to undergraduates? An article in the now-archived journal of Graduate Teaching Assistant Development recommended that giving a guest lecture on the scientific method was an excellent was for a GTA to gain experience, using the scientific method, "as illustrated with the GTA's own research (5)." GTAs would bring "confidence and enthusiasm to a presentation in which his/her research is a focal point." Indeed, we

recently found that a graduate student recently bringing in their thesis topic to lead a discussion section in introductory biology engaged both the GTA and undergraduates, and was a successful way to connect multiple course objectives into one lesson.

We recommend the following guidelines for bringing outside research into undergraduate discussion sections/lectures:

- Focus on a particularly difficult topic for students and incorporate several topics.
- Keep the course timeline in mind. What have students learned, what will they see later in the course, and what is beyond the scope of the course?
- Ask questions that relate directly to course learning objectives.
- Simplify graphs and/or figures. This may include giving information about what a measurement means, eliminating some groups from a graph/figure, or keeping to techniques that are either intuitive or have been previously discussed.
- Keep the presentation streamlined. Some details will most likely need to be omitted. (Ex. Students do not need to understand CRISPR to understand genetic mutations.)
- Live demonstrations or activities always help!

6.2 Exemplary Activity

The GTA developed this activity as a first-time TA for an introductory biology course for majors at a large university. Undergraduates attended lecture three times per week, and one discussion section per week led by a GTA. Teaching Assistants were encouraged to develop their own discussion section plan, with the stipulation that the 50-minute

discussion be mostly group work and active learning activities. The following is the GTA's account of bringing their thesis work into a discussion section.

"GTAs had access to clear learning objectives in advance, which assisted me greatly. The idea to include my thesis research came from my own uncertainty of how to teach a particularly difficult upcoming concept for students – membrane transport. My doctoral research focused on voltage-gated sodium channels and epilepsy, so I knew I could frame the discussion in this context. I was also able to use my research to address a common misconception for students.

As this discussion section came shortly before a midterm, I could also include previous course concepts such as protein structure, amino acid mutations, the scientific method, and graph reading.

Although a large portion of my doctoral research focuses on neuronal effects, I kept the course timeline in mind and omitted this part of the research from the discussion module. I also considered the introductory level of my students and simplified the graphs to ones they could reasonably understand.

Lastly, live demonstrations always help! One of the assays performed during my thesis research is a seizure behavior assay with Drosophila (S1). Flies are submerged in a warm water bath and monitored for seizure-like activity. This was a portable, easy to conduct, and easy to understand laboratory technique that I could bring to my discussions. Before the demonstration, the students and I discussed the set-up involved with the assay, including what variables were involved, and what blinding in research is. Then, in groups the students could observe the assay in real-time and identify what vial contained mutant

animals (those having seizure-like behavior). This illustration of how one amino acid change could alter membrane transport and result in organismal behavior was definitely the most popular part of discussion!"

Activity Guide

1. Before discussion, collect about 30 *Drosophila* each from one control line and one seizure mutant line into vials containing food. Bring the vials, a clear glass container of water, a hot plate, a thermometer, and mitts to discussion. In the classroom, fill the container about half-way with water, place on hot plate, and heat the water to about 40°C, but no hotter. (Omit this preparation if video S2 of assay will be used.)

2. Hand out the worksheet (S1) at the beginning of the discussion section. Have students work in groups of 3-5 on the first three questions, reviewing sodium transport and correcting a misconception about passive vs active transport. Then discuss as a class (10 minutes).

3. Have students work again in their groups on question 4, debating what amino acid changes would result in largest effects. Discuss as a class – although there is not a definite correct answer, focus should be on amino acid R groups' charges and shapes. (5 minutes)

4. Briefly review the overall goal of the fly seizure models and explain the seizure behavior assay based on referenced paper Schutte *et. al.* 2016 – one vial containing control flies and one vial containing seizure mutant flies would be dipped in the hot water bath. Scientists record the seizure assay over two minutes and later analyze the video for how much flies in each vial show seizure-like behavior over the two-minute trial (6). (5 minutes)

5. After the explanation, have students work on the remainder of the worksheet analyzing a graph of seizure behavior results from Schutte *et. al.* 2016. As a TA/instructor, transfer flies in the vials containing food to empty plastic vials – 2-3 vials with control flies and 2-3 vials with seizure mutants. Code the vials so only the TA/instructor knows which vial contains which flies. Discuss answers to worksheet questions 5-7. (10 minutes)

6. Invite students up to the experimental setup in smaller groups (1/2 to 1/3 of the class at a time) to view the seizure behavior assay. For each section, dip one fresh vial of control flies and one fresh vial of mutant flies in the hot water bath. (Or, show the video S2.) Students will observe seizure behavior in one vial and identify which vial contained the sodium channel mutants, an illustration of how an amino acid change can result in a behavioral defect. (5 minutes)

6.3 Module Worksheet

Bio93 Week 3 Discussion Section

How do Na+ ions cross the cell membrane? Circle all that apply.

Simple diffusion   *osmosis*   *ion channels*   *pumps*

If Na+ enters a cell, is it travelling down or up/against its electrochemical gradient? Why? How is this gradient established?

My lab studies a protein called SCN1A. This protein is a voltage-gated sodium channel found in neurons (nerve cells). When the channel opens, Na+ ions cross the membrane and enter the cell.

Is this an example of *active*   or *passive*   transport?

We are studying two different mutations in SCN1A which cause forms of childhood epilepsy, a disease characterized by recurrent seizures. Which mutation would you expect to cause a more severe form of epilepsy and why:

One in which the amino acid arginine is converted to cysteine?

One in which the amino acid arginine is converted to histidine?

Our lab studies 2 types of epilepsy:　　　　　GEFS+ (mild form)　　　　DS (severe form)

We model these epilepsies by studying fruit-flies we have created with protein mutations that match human patients. We hope to understand how the protein mutations affect sodium channel function, and how these epilepsies might be treated.

Human patients with GEFS+ or DS have febrile seizures, ones that occur during fevers when body temperature is high. We use hot water baths to simulate febrile conditions in flies, to see if flies with the same mutations in their sodium channels as human patients also have seizures at high temperatures.
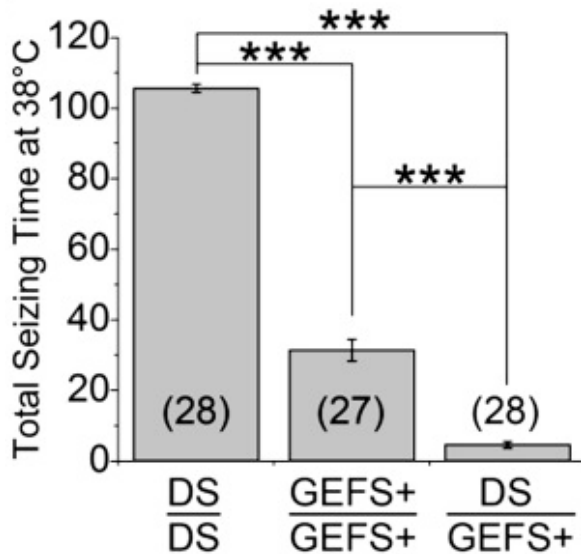
Flies with the DS mutation, GEFS+ mutation, or both mutations were observed for 120s in the hot water.

What additional data/group would you like to see shown on this graph?

Would you guess that the DS and GEFS+ mutations cause the same problem or different problems within the sodium channel and why?

Schutte, R.J., Schutte, S.S., Algara, J., *et. al.* (2014). Knock-in model of Dravet syndrome reveals a constitutive and conditional reduction in sodium current.

Our lab then isolates neurons to see how sodium mutations affect a cell's properties/activities. Once we know what goes wrong within the cell, we can maybe learn how to fix it!

6.4 Assessment and Expansion

Assessment

Undergraduates seemed highly engaged in this activity. During the quarter, students were invited to take an evaluation of their discussion TA. One question was open ended and asked, "what methods has this instructor used that have helped you to learn?" After compiling three years of student responses, one-eighth of students that participated in the survey *specifically* wrote-in this module as helpful. Quotes include, "she talked about her lab work and how that relates to what we are learning which is cool to see because it is applied," "bringing [her] flies with epilepsy was cool and very helpful in analyzing data", and "she brought in her own research to show us what we are learning right now is important because it will help us even after we complete our undergraduate studies." Many students also emailed the GTA after this week, wondering about undergraduate involvement in research on campus. This is another key benefit of bringing campus research into the classroom. Every student in the discussion sections had the opportunity to learn about, ask questions about, and physically view scientific research happening on campus. Many of the students in introductory biology are first-generation and may not have been aware undergraduate research was even a possibility, much less be aware of how much undergraduate research helps for graduate or medical school applications. Early exposure can bring awareness to more students for their consideration and planning. The more comfortable graduate student-undergraduate relationship may also permit students to ask graduate students about the best way to find a lab and contact professors, before undergraduates contact said faculty members.

This activity was enjoyable, engaging, and memorable for undergraduates, but was not solely for their benefit. The GTA can benefit great as well. In the words of the GTA who put together the activity, "As a novice graduate student, I was much more confident for this week of discussion than other weeks. During the planning process, I learned how to carefully consider course/learning objectives and tie multiple concepts together. I also learned how to explain my thesis in an understandable way, a skill that also helped me as a researcher. I highly recommend graduate teaching assistants to use their thesis research to lead a discussion, for the benefit of both TAs and undergraduates."

Expansion

The activity was quite successful and worked well for both the undergraduates and the GTA. The GTA used her transgenic fly models again for two more topics – central dogma (S3) and (briefly) action potentials.

Central dogma is an easy place to incorporate any transgenic model. Students had already learned about amino acid mutations in a sodium channel resulting in epilepsy. In this second exposure, students transcribed and translated a section of the Drosophila sodium channel gene to investigate how amino acid substitutions arose from base pair changes. Students then looked at example figures to determine if/how differences in amino acid sequence could be visualized in the lab (northern blot, Southern blot, western blot).

The final topic covered in the introductory biology course was neurons and action potentials. Again, the GTA used her mutant flies as an example – where in the neuron would these sodium channels be located, and how would a change in sodium ion influx influence the action potential? This third exposure was the first time students would learn the

specifics of the mutation's effects at the cellular level. The intention was that repeated exposures to the same example mutation using different lenses would help illustrate the importance of the central dogma and tie together concepts learned in different weeks of the course. It also made it easier for the GTA to develop new discussion material each week.

Simplifying real-world research into activities for undergraduates can engage both graduate students and undergraduates, provide context for and a connection of several learning objectives at once, provide opportunities for active learning including engaging with figures, and pique interest in on-campus research. We highly encourage GTAs to incorporate their thesis research into discussion sections, to aid in both GTA and undergraduate engagement, and their appreciation of the connection between principles in a textbook and real world application.

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CHAPTER VII

Discussion and Significance

7.1 Discussion

Fruit flies serve as fruitful models for neurological diseases

In order for a model system of human disease to be accepted, the model frequently needs to show validity in a few ways: 1. Does the model 'look like' the human disease or recapitulate symptoms associated with a disease? 2. Are the model's phenotypes due to similar mechanisms (genes, environmental causes, toxins) as the human disease? 3. Can the model's phenotypes be altered (with drugs, environmental factors) to make predictions regarding the human disease? With over one hundred years of genetic studies in *Drosophila melanogaster*, the unparalleled genetic tools since developed, and functional orthologs to about 75% of disease-related genes in humans, the lowly fruit fly has proven to be an efficient and genetically tractable model for studying human diseases (Bellen et. al. 2010, Reiter et. al. 2011, Narayan and Rothenfluh 2015). In addition, the Drosophila nervous system is organized similarly to the vertebrate brain, shares developmental principles and key proteins, but is less complex (Sanes and Zipursky 2010, McGurk et. al. 2015), making Drosophila a particularly useful model for neurodegenerative and neuropsychiatric diseases (Bilen and Bonini 2005, Rosch et. al. 2019). Indeed, Drosophila have provided useful models of human neurological disorders including Parkinson's disease, Fragile X syndrome, and spinocerebellar ataxia, among many others (Feany and Bender 2000, Dockendorff et. al. 2002, Mutsuddi et. al. 2004).

Fruit flies have also been fruitful in the study of epilepsy disorders. Traditionally, Drosophila models involved forward genetic screens to identify animals with mutations that exhibited desired behavioral alterations. In the 1970s, the search was on for "mutations

affecting nerves and/or muscles" for further study (Suzuki et. al. 1971). These forward screens led to mutants with heat-induced paralysis, *para^ts* (Suzuki et. al. 1971, Siddiqi and Benzer 1976), and mechanically induced seizures (Ganetzky and Wu 1982). The genetic basis for *para^ts* was later mapped to a voltage-gated sodium channel, then named Para (Loughney et. al. 1989). One of the bang-sensitive (BS) mutants was also mapped to *para* L1699F (Parker et. al. 2011). BS lines were the first main Drosophila model of epilepsy, helped validate the model by demonstrating the effectiveness of common antiepileptic drugs on the flies (Kuebler and Tanouye 2002, Reynolds et. al. 2004) and continue to be used to explore factors of seizure susceptibility, pathways involved in seizure generation and screen potential antiepileptic drugs (Kuebler et. al. 2001, Bao et. al. 2011 Giachello and Baines 2015, Lin et. al. 2017, Dare et. al. 2021).

Bang-sensitive flies are an important seizure model, however, to specifically examine how individual mutations in identified genes lead to human diseases requires the ability to make animals with precisely targeted mutations. The mammalian voltage-gated sodium channel gene SCN1A is a hotspot for epilepsy-causing mutations, yet the connection between individual mutations and specific patient symptoms remains elusive (Catterall et. al. 2010, Escayg and Goldin 2015). The Drosophila voltage-gated sodium channel gene (*para*) encodes a protein (Para) similar in structure and function to $Na_V1.1$ (O'Dowd et. al. 1989, Loughney et. al. 1989). Reverse genetics has resulted in Drosophila seizure models that share more similarities to human mechanisms of seizure disorders.

Creation of Drosophila knock-in models of epilepsy

Our lab previously used ends-out homologous recombination (Gong and Golic 2003, Staber et. al. 2011) to generate the first knock-ins of epilepsy in Drosophila with mutations

in para homologous to patients with known SCN1A mutations K1270T (Sun et. al. 2012) and S1231R (Schutte et. al. 2014). K1270T leads to the moderate seizure disorder GEFS+ in humans (Abou-Khalil et. al. 2001), and results in heat-induced seizures, increased sodium currents, and reduced inhibition in Drosophila (Sun et. al. 2012), as well as sleep disturbances (Petruccelli et. al. 2015), and amelioration of symptoms with lithium (Kaas et. al. 2016). S1231R leads to the severe seizure disorder Dravet Syndrome in humans (Fujiwara et. al. 2013), and in flies, results in seizures in heat-induced seizures of greater severity than those in K1270T flies, decreased sodium current, reduced inhibition, and amelioration of symptoms with serotonin (Schutte et. al. 2014). S1231R and K1270T knock-in Drosophila demonstrated that fruit flies can be used to model genetic human epilepsies, retaining genetic homology and salient behavioral phenotypes while allowing for exploration of seizure generation mechanisms at a single-cell level.

Since distinct behaviors in humans corresponded with distinct behavioral and mechanistic phenotypes in flies as well, we were interested in the possibility of using many mutant *Drosophila* lines to further model and understand different patient-specific epilepsy-causing mutations. To better understand the diversity and connection between mutations and mechanisms of seizure generation, my thesis project focused on two missense mutations occurring at the same residue within SCN1A but leading to different epilepsy diagnoses. Recent advances led us to try a new gene editing technology, CRISPR/Cas9. In 2012, the Charpentier and Doudna labs worked together to harness the DNA-cutting scissors of bacteria's immune system, opening the door to precise genome editing (Jinek et. al. 2012). The next year, researchers were already optimizing the use of CRISPR/Cas9 in Drosophila, including the recommendations for embryo injection of repair DNA and using germ-line Cas9

for efficient transmission (Bassett et. al. 2013, Gratz et. al. 2013, Ren et. al. 2013, Yu et. al. 2013, Sebo et. al. 2014, Gratz et. al. 2015). Now there are even guides for the use of tissue-specific CRISPR in Drosophila (Meltzer et. al. 2019, Port et. al. 2020).

While homologous recombination worked for our previous studies, the technology depends on homologous recombination events that naturally occur at low frequency. To increase efficiency for our concurrent studies on R1648H and R1648C in Drosophila, we made an initial one-step CRISPR attempt following recommended guidelines. Pre-blastoderm embryos of Drosophila expressing germline vas-Cas9 were injected with CRISPR guide RNA (gRNA) to generate a double stranded break 4 nucleotides downstream of the PAM sequence, and a 50-nucleotide single stranded oligodeoxynucleotide repair template containing the desired mutation. The selection marker was a *TaqI* cut site interrupted by CRISPR. In order to find successfully transformed lines, PCR and gels needed to be run for every single fly, once they were crossed. While this technique theoretically could have worked, it was onerous, and though DNA from 2000 flies was extracted and gel analyzed, none had a visibly disrupted Taq1 site!

A second, successful, strategy involved a repair plasmid of visible marker DsRed, expressed in eyes, to replace the entirety of exon 30, where the codon for residue 1648 of SCN1A is located. Only flies screened for the visible marker were used for a second CRISPR injection to replace DSRed with a transgenic exon 30. While *para* is the only sodium channel gene in the fly, the introduction of worm sequences flanking the replacement sequence in the first step for targeted excision in the second step further decreased any concerns about off-target effects, as the *C. elegans* DNA sequences are not found in the Drosophila genome.

This resulted in efficient detection of multiple founder flies with the desired mutations, and no unexpected mutations. Beyond demonstrating proof-of-concept, the intermediary line for these studies could serve as an intermediary to make many other mutant lines, specifically other mutations in exon 30. This includes mutations in residues 1619 to 1719 spanning the end of DIVS3 through part of DIVS5, of which about 120 have been identified in SCN1A registries - the majority of these missense mutations, and a large number attached to a DS diagnosis (scn1a.caee.org, Ishi et. al. SCN1A.net). A larger pool of mutants with the same genetic background would allow for further exploration of the heterogeneity or consistency of cellular changes induced by SCN1A/para mutations.

Conclusions drawn from R1648C and R1648H Drosophila

R1648C and R1648H encode Dravet Syndrome and GEFS+, respectively. CRISPR/Cas9 generated R-C and R-H Drosophila displayed generally similar heat-exacerbated seizure-like behavior, reduced lifespan, and alterations in circadian activity and sleep. Mutant inhibitory neurons from adult Drosophila exhibited similar sustained depolarizations and reduced firing frequency, with a constitutively hyperpolarized sodium current deactivation threshold.

There were no dramatic differences in excitatory neurons between R-C, R-H, and R-R flies, suggesting that a loss in inhibition was the main contributor to hyper-excitability in the mutants' neural network. In K1270T in Drosophila, an increase in the firing frequency of excitatory neurons was observed in concert with a decrease in firing of inhibitory neurons, and sustained depolarizations in inhibitory neurons were not seen in excitatory neurons. Though R1648H and R1648C local neurons shared many cellular phenotypes to K1270T LNs,

particularly decreased inhibitory firing and sustained depolarizations excluded from projection neurons (PNs), R1648 LNs did not show the corresponding increase in excitatory PN firing (Sun et. al. 2012, Schutte et. al. 2016). In fact, our preliminary studies on PNs in R1648H and –C flies found no apparent differences between mutants and controls. This is similar to PNs from S1231R Drosophila, though the findings in R1648H and –C LNs much more closely resemble the previous K1270T flies than the S1231R Drosophila (Schutte et. al. 2014, Schutte et. al. 2016). In K1270T iPSC-derived neurons, a reduced sodium current density in excitatory neurons was not sufficient to reduce excitatory firing, though a reduction in sodium current density in inhibitory neurons did contribute to reduced inhibitory firing (Xie et. al. 2020). These findings continue to suggest that inhibitory neurons seem to be most impacted by SCN1A mutations, and also that there is diversity in the possible relationships between inhibitory and excitatory cellular phenotypes within a model.

The similarity of the behavioral and cellular phenotypes in the different mutant fly lines suggests that disorders of different severity in humans could be due in large part to sharing the same location in mutation, and differences in genetic background. The finding that fly lines homozygous for the R-C or R-H mutations were lethal, and that heterozygotes displayed significant seizure activity and reduced lifespan, was somewhat unexpected. In studies of the previous S1231R and K1270T Drosophila lines, alterations in sodium current and firing properties were apparent only in animals homozygous for the mutations. Heterozygotes exhibited moderate seizure behavior at high temperatures but no obvious alterations in neuronal activity (Sun et. al. 2012, Schutte et. al. 2014). The location of the 1648 mutation in DIVS4, the voltage-sensing region of *SCN1A* particularly crucial for inactivation, likely explain this increased level of severity compared to the previously

examined S1231R mutation in DIIIS1, and K1270T mutation in DIIIS2. Both R-C and R-H mutations likely impact the ability of the S4 segment in domain IV to move back into place, closing the channel (Ahern et. al. 2016, Pan et. al. 2019). As sodium channels are electronically distant from the soma of LNs and PNs recorded from in our studies, it would be difficult to draw conclusions about the kinetics of activation and inactivation in the Drosophila models. Further studies in iPSC-derived neurons for function, and cryogenic electron microscopy for structure, could better address functional changes to the sodium channel on the molecular level.

Mutation location alone does not explain the heterogeneity in symptoms of those with epilepsy-causing SCN1A mutations. For example, R1648H was identified in thirteen individuals from the same family whose symptoms ranged from experiencing a few febrile seizures in early childhood to intractable epilepsy throughout adulthood associated with moderate or severe intellectual disability, with differential drug responses (Baulac *et. al.* 1999). This suggests interplay between the mutation and other factors are involved in determining disease severity. To learn more about genetic modifiers contributing to seizure susceptibility, Drosophila obtained from mutagenized screens could be crossed to R-H/R-C Drosophila or other known mutants. A seizure behavior assay could serve as an easy screen for a phenotypic change. Double mutants with phenotypic change could be further studied to identify candidate genes involved. Additionally, future work on iPSC-derived neurons from different patients with the same mutation could aim to root out some of these genetic modifiers.

Uncovering pathways affecting seizures in Drosophila

Drosophila, with their simple behavioral assays and high-throughput system, could be well suited for drug studies, as well as double mutant screens. We developed a potential list of drugs to determine whether the symptoms of epilepsy can be lessened in R1648C and R1648H flies with various compounds. The results of which drugs reduce or exacerbate seizures in our flies could inform us about which cellular mechanisms or pathways were at play. The drug candidates chosen included avobenzone, ranolazine, serotonin precursor 5-HTP, and an endocannabinoid.

Dr. Richard Baines' lab found that avobenzone increases amounts of *pumilio* in a bang-sensitive fly model of epilepsy. Pumilio was previously shown to bind to mRNA (including that of sodium channel genes) and interrupt translation. This effectively reduced seizure recovery time for bang-sensitive flies as they contain a gain-of-function mutation in their para sodium channel. Pumilio-mediated regulation of translation decreases the effect of extra sodium channel activity caused by the mutation (Lin et. al. 2017). We also sought to test serotonin, shown in our lab's previous work to exacerbate seizures in a K1270T GEFS+ Drosophila model (Schutte et. al. 2014) but ameliorate symptoms in S1231R DS Drosophila (Sun et. al. 2012), ranolazine, which selectively blocked aberrant persistent current in a heterologous expression system model of R1648H and C (Kahlig *et al.* 2010), and cannabinoids (Jimenez-Del-Rio *et al.* 2008), found to reduce seizure-like behaviors in patients and animal models of SCN1A epilepsies. Any drug screens with remarkable results had the potential to lead to further electrophysiological studies.

Adapted from Lin et. al. 2017, groups of newly-eclosed flies fed for 24 hours on filter papers soaked in a sucrose/vehicle/drug solution (without drug for the controls). The vehicle was DI water for ranolazine and 5-HTP. The vehicle was DMSO for avobenzone. For the avobenzone trials, the vials were wrapped in foil, as both avobenzone and its vehicle, DMSO, are light sensitive. For hydrophobic endocannabinoid, the drug was mixed into standard cornmeal-based food and provided that way. After 24 hours, the flies were subjected to the two-minute seizure behavior assay at 40°C described earlier. Drugs were tested at concentrations of 0.5, 5.0, and 50mM. Although electrophysiological results strongly suggest that both R-C and R-H mutations result in gain-of -sodium channel activity and we believed the effects of gain-of-function mutations would be ameliorated by avobenzone or ranolazine in particular, the presence of any compound tested did not improve seizure susceptibility in our mutants.

To confirm the flies were ingesting avobenzone and other drugs, we set up an experimental round overnight with blue dye in the drug solution (DMSO vehicle, 5% sucrose, avobenzone) and in 24 hours when checked, the presence of blue was confirmed in the flies' abdomens. The genotype of flies initially tested in Baines' lab were bang-sensitive mutants. These flies do not contain a patient-specific mutation and do not mimic patients' heat-sensitive seizures, being instead mechanosensitive. Additionally, the cells in the *bss* flies used for electrophysiology were larval motor neurons. Although an abnormally large persistent current these neurons was associated with the bss flies, the bss and SCN1A mutant flies may have different mechanisms of dysfunction in their respective central neurons.

Additionally, there may be pharmacological differences between *para* and the mammalian sodium channel. 75% of SCN1A residues that are mutation locations are conserved in Drosophila, and another 15% are similar (Lossin et. al. 2009). While the voltage-sensing S4 segments of *para* are 80% conserved with respect to human SCN1A, *para* as a whole is 42% identical to SCN1A based on standard alignment analysis (Uniprot.org). The structure for toxin or other pharmacological binding sites may differ between mammalian and invertebrate sodium channels. Even without conservation of all binding sites, drug studies in Drosophila can inform us about pathways involved in seizure susceptibility. The previous use of 5-HTP in Drosophila models is an example of the benefits of this work. One model, S1231R, had reversible suppression of symptoms with after 3 days of 5-HTP ingestion (Schutte et. al. 2014). Acute serotonin injection also reduced seizures in S1231R animals, but the effect was considerably smaller than with 5-HTP feeding, suggesting that a more longterm pathway might be involved. Combined with successful clinical studies using serotonin reuptake inhibitors to treat refractory epilepsies (Favale et. al. 2003, Albano et. al. 2006), the data suggests the serotonin pathway as a potential target for understanding and treating epilepsies, particularly DS.

The electrophysiological studies performed on knock-in Drosophila models of epilepsy have occurred mainly at the antennae lobe (AL). While we do not know the locations of seizure generation within Drosophila, the AL is a central circuit within the Drosophila brain that is characterized to the individual cell level and analogous to the vertebrate olfactory bulb (Horne et. al. 2018, Liou et. al. 2018). About 1300 olfactory sensory neurons of 50 different classes converge onto 50 glomeruli within the AL, transmitting to ~150 excitatory projection neurons that terminate in the higher processing areas of the mushroom

body or lateral horn. Connecting the network is ~200 inhibitory local neurons, which are heterogeneous in class themselves (Vosshall and Stocker 2007, Chou et. al. 2010, Seki et. al. 2010, Wilson et. al. 2013, Nagel and Wilson 2016). Using inhibitory and excitatory neurons from the same circuit for analysis contributed to an understanding of the balance of excitation within the brain. As the only Drosophlia sodium channel, Para, is expressed diffusely, other populations of cells could be studied, for example the glutamatergic motor neurons of the adult thoracic ganglion (Schutte et. al. 2016), Kenyon cells of the mushroom body involved in odor processing and olfactory memory (Owald and Waddell 2015), or the large ventral lateral clock neurons used in the study of sleep and circadian activity (Sheeba et. al. 2008). Additionally, any functional deficits caused by altered cellular properties in the antennae lobe could be explored with assessments of learning and memory in R-C or R-H Drosophila (Kahsai and Zars 2011). Finally, specific GAL4/UAS lines could be used to express mutations in targeted areas of the brain to explore how different areas of the Drosophila brain contribute to seizure generation or other behavioral symptoms.

7.2 Significance

We used a novel two-step CRISPR/Cas9 gene editing protocol to generate two Drosophila lines with *para* sodium channel mutations homologous to human sodium channel SCN1A-mediated epilepsy causing mutations, and one transgenic control line. This strategy facilitated exploration of how two mutations at one residue led to distinct epilepsy disorders, and opens the door for the easy creation of additional mutant lines in the same region of SCN1A. Transgenic Drosophila display phenotypes resembling human phenotypes of epilepsy, including heat-exacerbated seizures, premature death, and aberrant sleep/wake cycles. We refined a protocol to record from single cells in the adult Drosophila brain to

explore both neuronal firing and sodium current properties. Antennae lobe neurons from adult mutant flies displayed interrupted inhibition but not interrupted excitation, tipping the circuit balance in favor of overexcitation and further supporting the idea that SCN1A mutations mainly affect inhibitory neurons. The findings that distinct mutations at the same position within SCN1A in the same genetic background lead to similar phenotypes further provides insight that mutation location and genetic modifiers play a crucial role in patient symptoms and responses to treatment. The transgenic Drosophila lines and the seizure behavior assay utilized in this thesis work also contributed to the development of a module for an undergraduate discussion session illustrating the shared benefit to both teaching assistant and undergraduate students of using outside research in the classroom.

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