MECHANISMS OF SOUTHERN GRASSHOPPER MOUSE (*ONYCHOMYS TORRIDUS*) MUSCLE RESISTANCE TO THE PARALYTIC AND LETHAL TOXINS IN ARIZONA BARK SCORPION (*CENTRUROIDES SCULPTURATUS*) VENOM

By

Abhijna A. Parigi

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ABSTRACT

MECHANISMS OF SOUTHERN GRASSHOPPER MOUSE (*ONYCHOMYS TORRIDUS*) MUSCLE RESISTANCE TO THE PARALYTIC AND LETHAL TOXINS IN ARIZONA BARK SCORPION (*CENTRUROIDES SCULPTURATUS*) VENOM

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Voltage-gated sodium ion channels (Na_v) are transmembrane proteins responsible for initiating electrical signals in excitable cells. Because Na_v channels play a crucial role in neuromuscular coordination, they are targeted by a diverse array of neurotoxins produced across the animal kingdom. Arizona bark scorpions (*Centruroides sculpturatus*) produce toxins that disrupt Na_v channel function, causing pain, muscle paralysis and respiratory failure. Southern grasshopper mice (*Onychomys torridus*) hunt bark scorpions. In response to selection by scorpion venom, grasshopper mice have evolved physiological resistance to toxins that cause pain and death. Although previous work identified modifications in one grasshopper mouse Na_v channel ($Na_v1.8$) that provide resistance to venom pain, mechanisms underlying resistance to muscle paralysis remain unknown. In skeletal muscle, $Na_v1.4$ channels regulate muscle contraction. Previous studies showed that toxins in *C. elegans* and *C. vittatus* venoms disrupt $Na_v1.4$ gating mechanisms. Thus, I tested the hypothesis that *C. sculpturatus* venom contains toxins that target $Na_v1.4$, and that grasshopper mice are resistant to the effects of these toxins via molecular changes to their $Na_v1.4$. Using molecular and electrophysiological analyses, I compared the structural and functional properties of grasshopper mice $Na_v1.4$ channels to those of house mice and rats (rodents that are both sensitive to scorpion venom) and found that grasshopper mice $Na_v1.4$ has evolved

reduced sensitivity to *C. sculpturatus* venom. Further, I identified amino acid changes in the grasshopper mice $Na_v1.4$ protein that contribute to reduced toxin sensitivity. Finally, I show that the beta subunits (accessory proteins that modulate $Na_v1.4$ channel gating kinetics) of grasshopper mice do not have species-specific effects on channel function in the presence or absence of venom. My results demonstrate that highly conserved proteins can be evolutionarily modified with minimal effects to their baseline functional properties.

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KEY TO ABBREVIATIONS

ANOVA	Analysis of Variance
BSA	Bovine Serum Albumin
cDNA	Complementary Deoxyribonucleic Acid
CIP	Calf Intestinal Phosphate
cRNA	Capped Ribonucleic Acid
D	Domain
GaGb	Grasshopper mouse Alpha subunits co-expressed with Grasshopper mouse Beta subunits
GaRb	Grasshopper mouse Alpha subunits co-expressed with House mouse Beta subunits
GaRb	Grasshopper mouse Alpha subunits co-expressed with Rat Beta subunits
K_d	Dissociation Constant
Na^+	Sodium
Na_v	Voltage-Gated Sodium Ion Channel
OCB	Open Channel Blocker
PAML	Phylogenetic Analysis using Maximum Likelihood
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
S	Segment
TTX	Tetrodotoxin

INTRODUCTION

A central goal in evolutionary biology is to understand the molecular mechanisms underlying adaptive traits. Given that predator-prey interactions over evolutionary time have resulted in some of the most specialized adaptations (Brodie & Brodie 1990; Miyatake *et al.* 2004; Gosline & Rodd 2007; Stuart-Fox *et al.* 2008; Voss & Jansa 2012; McCabe *et al.* 2016), predators and their prey can serve as ideal models for understanding adaptive traits and the molecular processes that produce them. **My research uses defensive neurotoxic venoms and their ion channel targets in predators as a model to study the molecular and physiological bases of neuromuscular adaptations.**

The survival of both predators and prey in the wild depends on their ability to detect and respond to environmental cues. In animals, the neuromuscular system coordinates stimulus detection and response via specialized proteins called voltage-gated sodium (Na_v) ion channels (Hodgkin & Huxley 1952; Marban *et al.* 1998; Yu & Catterall 2003). Given the importance of these channels in mediating all vital activities (e.g. obtaining food, navigation, detecting mates), neuromuscular systems in general, and Na_v channels in particular are conserved across diverse taxa (Goldin 2002; Meisler 2005). Consequently, some of the most effective offensive and defensive traits are those that target and manipulate Na_v channels, ultimately disrupting neuromuscular activity (Possani *et al.* 1999; Al-Sabi *et al.* 2006; Wingerd *et al.* 2017).

An excellent example of a Na_v channel-manipulating trait is neurotoxic venom produced by species of scorpion in the genus *Centruroides* (Kirsch *et al.* 1989; Gordon *et al.* 1996; Cestèle *et al.* 1999; Possani *et al.* 1999; Campos *et al.* 2008; Zhang *et al.* 2015). Commonly known as “bark scorpions”, these small arthropods (approximately 0.5 –1.0 gram) defend themselves from potential predators by inflicting toxic stings that can cause pain, paralysis and death (Patterson 1960; Rimsza *et al.* 1980; LoVecchio & McBride 2003). While we would predict most predators to simply avoid hunting bark scorpions, one predatory rodent, the grasshopper mouse (*Onychomys spp.*) is known to hunt and consume bark scorpions in the wild. Previous research in our lab has shown that grasshopper mice have evolved physiological resistance to venom (Rowe & Rowe 2008) through modifications to at least one Na_v channel (Rowe *et al.* 2013). Such an evolutionary solution is astounding, as even a few amino acid changes to highly conserved proteins can cause devastating diseases in humans (Hoffman *et al.* 1995; Vicart *et al.* 2005; Drenth & Waxman 2007; Struyk *et al.* 2008; Webb 2009; Jurkat-Rott *et al.* 2010; Dib-Hajj *et al.* 2012; Groome *et al.* 2014). In this study, my aim was to understand the molecular mechanisms underlying venom resistance in grasshopper mice, and the potential physiological costs that might arise from modifications to important proteins.

Voltage-gated Na_v channels, transmembrane protein complexes expressed in nerve and muscle cells, mediate electrical signals to and from the brain by regulating the flow of charged Na^+ ions (McCleskey & Gold 1999; Goldin 2001; Wood & Baker 2001; Wood *et al.* 2004). Each Na_v channel is made of one large pore forming alpha subunit (~270kD) and two accessory proteins called beta subunits (32-35 kD); one beta

subunit binds covalently with the Na_v alpha subunit, while the other associates non-covalently (Catterall 2000; Winters & Isom 2016). Mammals express 9 different types of Na_v alpha subunits and at least 4 types of beta subunits (beta 1- beta 4), all of which are expressed with distinct tissue specificity (Isom *et al.* 1992; Isom *et al.* 1995; Yu & Catterall 2003; Patino & Isom 2010; Brackenbury & Isom 2011; Isom 2016; Kruger & Isom 2016). Previous studies showed that toxin peptides in the venom of some species of *Centruroides* bind to specific regions on the alpha subunit of Na_v channels, causing the channels to activate prematurely (near resting membrane potential), and delay fast inactivation (Kirsch *et al.* 1989; Gordon *et al.* 1996; Cestèle *et al*. 1998; Mantegazza & Cestèle 2005; Campos *et al.* 2008). Some peptides target Na_v channels of pain-sensing neurons, while others bind to Na_v channels in muscle and cause paralysis (Watt 1990; LoVecchio & McBride 2003). When stung by the Arizona bark scorpion, small mammals (and human infants) often die from suffocation due to paralysis of their diaphragm (Rimsza *et al.* 1980; LoVecchio & McBride 2003).

Three species of grasshopper mice, *O. torridus, O. arenicola, O. leucogaster*, are carnivorous, desert-dwelling rodents of Southwestern United States and Mexico (Riddle & Honeycutt 1990) that regularly hunt bark scorpions (Rowe *et al.* 2006; Rowe *et al.* 2008). Grasshopper mice are undeterred by bark scorpion stings, having evolved physiological resistance to the venom peptides (Rowe & Rowe 2008). Previous work in the Rowe lab showed that one Na_v channel isoform expressed in the pain pathway of grasshopper mice ($Na_v1.8$) has amino acid substitutions that allow the channel to bind venom peptides and block pain signals - put simply, grasshopper mice use venom as an anesthetic (Rowe *et al.* 2013). However, we still do not understand how grasshopper

mice can tolerate peptides that cause paralysis and death. Preliminary data obtained from whole muscle and nerve preparations suggested that the muscles of grasshopper mice are resistant to *C. sculpturatus* venom. Whereas the muscles of house mice exhibited a temporary venom-induced increase in the force of muscle contraction followed by a block of contraction, grasshopper mice muscles exhibited the venom-induced increase in force of contraction only at high concentrations of venom. Moreover, muscle contraction was never blocked in grasshopper mice, even when the muscle was exposed to high concentrations of venom for prolonged periods of time (> 8 hours, A. Rowe data unpublished). Additionally, previous work (Rowe *et al.* 2011; Vandendriessche *et al.* 2010) showed that isolated peptides from the venoms of *C. vittatus* and *C. elegans* (respectively) target $Na_v1.4$ channels expressed in heterologous systems. **My hypothesis is that the *C. sculpturatus* venom contains peptides that alter the gating function of $Na_v1.4$, and that the grasshopper mouse $Na_v1.4$ channel complex (alpha and beta subunits) has evolved structural and functional modifications that impart resistance to these peptides.**

In the first chapter of my dissertation, I demonstrate that *C. sculpturatus* venom prematurely activates and then delays inactivation in $Na_v1.4$ channels from *Mus musculus* (house mice) and *Rattus norvegicus* (rats) (control species known to die from scorpion envenomation). In comparison, grasshopper mouse $Na_v1.4$ was less sensitive to the effects of *C. sculpturatus* venom. Using models of molecular evolution and mutagenesis studies, I identified the specific amino acid modifications involved in conferring reduced sensitivity to venom.

In Chapter 2, I examined the role of grasshopper mouse accessory beta subunits in modulating $Na_v1.4$ baseline functional properties and response to *C. sculpturatus* venom. My results show that the grasshopper mouse beta subunits do not have any special modulatory effects on their alpha subunits either in the presence or absence of venom.

REFERENCES

REFERENCES

Al-Sabi A, McArthur J, Ostroumov V & French RJ. 2006. Marine toxins that target voltage-gated sodium shannels. *Marine Drugs* 4(3):157–192.

Brodie EDI & Brodie EDJ. 1990. Tetrodotoxin resistance in garter snakes: an evolutionary response of predators to dangerous prey. *Evolution* 44(3):651–659.

Campos FV, Beirão PSL & Bezanilla F. 2008. Alpha-scorpion toxin impairs a conformational change that leads to fast inactivation of muscle sodium channels. *The Journal of General Physiology* 132(2):251–263.

Catterall WA. 2000. From ionic currents to molecular mechanisms: the structure and function of voltage-gated sodium channels. *Neuron* 26(1):13–25.

Cestèle S, Stankiewicz M, Mansuelle P, De Waard M, Dargent B, *et al.* 1999. Scorpion alpha-like toxins, toxic to both mammals and insects, differentially interact with receptor site 3 on voltage-gated sodium channels in mammals and insects. *The European Journal of Neuroscience* 11(3):975–985.

Cestèle S, Qu Y, Rogers JC, Rochat H, Scheuer T *et al.* 1998. Voltage sensor-trapping: enhanced activation of sodium channels by beta-scorpion toxin bound to the S3-S4 loop in domain II. *Neuron* 21(4):919–931.

Dib-Hajj SD, Yang Y, Black JA & Waxman. 2012. The Na(V)1.7 sodium channel: from molecule to man. *Nature Reviews Neuroscience* 14(1):49-62.

Drenth JPH & Waxman SG. 2007. Mutations in sodium-channel gene SCN9A cause a spectrum of human genetic pain disorders. *Journal of Clinical Investigation* 117(12):3603–3609.

Goldin AL. 2002. Evolution of voltage-gated Na(+) channels. *The Journal of Experimental Biology*, 205(5):575–584.

Goldin AL. 2001. Resurgence of sodium channel research. *Annual Review of Physiology* 63(1):871–894.

Gordon D., Martin-Eauclaire MF, Cestèle S, Kopeyan C, Carlier R, *et al.* 1996. Scorpion toxins affecting sodium current inactivation bind to distinct homologous receptor sites on rat brain and insect sodium channels. *The Journal of Biological Chemistry*, 271(14):8034–8045.

Gosline AK & Rodd FH. 2007. Predator-induced plasticity in guppy (*Poecilia reticulata*) life history traits. *Aquatic Ecology* 42(4):693–699.

Groome JR, Lehmann-Horn F, Fan C, Wolf M, Winston V, *et al.* 2014. $Na_V1.4$ mutations cause hypokalaemic periodic paralysis by disrupting IIIS4 movement during recovery. *Brain* 137(4):998–1008.

Hodgkin, AL & Huxley AF. 1952. A quantitative description of membrane current and its application to conduction and excitation in nerve. *The Journal of Physiology*, 117(4):500–544.

Hoffman EP, Lehmann-Horn F & Rädel R. 1995. Overexcited or inactive: ion channels in muscle disease. *Cell* 80(6):681-686.

Patterson RA. 1960. Physiological action of scorpion venom. *American Journal of Tropical Medicine and Hygiene* 9(4):410–414.

Isom LL, De Jongh KS, Patten DE, Reber BF, Offord J, *et al.* 1992. Primary structure and functional expression of the beta 1 subunit of the rat brain sodium channel. *Science* 256(5058):839–842.

Isom LL. 2016. Sodium channel β subunits: anything but auxiliary. *The Neuroscientist* 7(1):42–54.

Isom LL, Ragsdale DS, De Jongh KS, Westenbroek RE, Reber BF, *et al.* 1995. Structure and function of the β2 subunit of brain sodium channels, a transmembrane glycoprotein with a CAM motif. *Cell* 83(3):433–442.

Brackenbury WJ & Isom LL. 2011. Sodium Channel β1 Subunits: Overachievers of the Ion Channel Family. *Frontiers in Pharmacology* 2:53.

Jansa SA, & Voss RS. 2011. Adaptive evolution of the venom-targeted vWF protein in opossums that eat pitvipers. *PLoS ONE*, *6*(6): e20997.

Jurkat-Rott K, Fauler M, Lehmann-Horn F. 2010. Sodium channelopathies of skeletal muscle result from gain or loss of function. *Pflügers Archiv* 460(2):239–248.

Kirsch GE, Skatterbøl A, Possani LD & Brown AM. 1989. Modification of Na channel gating by an alpha scorpion toxin from *Tityus serrulatus*. *The Journal of General Physiology* 93(1):67–83.

Kruger LC & Isom LL. 2016. Voltage-gated Na^+ channels: not just for conduction. *Cold Spring Harbor Perspectives in Biology* 8(6):a029264–15.

LoVecchio F & McBride C. 2003. Scorpion envenomations in young children in central Arizona. *Journal of Toxicology: Clinical Toxicology* 41(7):937–940.

Mantegazza M & Cestèle S. 2005. β-Scorpion toxin effects suggest electrostatic interactions in domain II of voltage-dependent sodium channels. *The Journal of Physiology* 568(1):13–30.

Marban E, Yamagishi T & Tomaselli GF. 1998. Structure and function of voltage-gated sodium channels. *The Journal of Physiology* 508(3):647–657.

McCabe TM & Mackessy SP. 2016, Evolution of resistance to toxins in prey. In: Gopalakrishnakone P & Malhotra A (eds). *Evolution of venomous animals their toxins. Toxinology.* Springer, Dordrecht, Netherlands.

McCleskey EW & Gold MS. 1999. Ion channels of nociception. *Annual Review of Physiology* 61:835–856.

Meisler MH. 2005. Sodium channel mutations in epilepsy and other neurological disorders. *Journal of Clinical Investigation* 115(8):2010–2017.

Miyatake T, Katayama K, Takeda Y, Nakashima A, Sugita A, *et al*. 2004. Is death-feigning adaptive? Heritable variation in fitness difference of death-feigning behaviour. *Proceedings of the Royal Society B: Biological Sciences* 271(1554):2293–2296.

Patino GA & Isom LL. 2010. Electrophysiology and beyond: multiple roles of Na^+ channel β subunits in development and disease. *Neuroscience Letters* 486(2):53–59.

Possani LD, Becerril B, Delepierre M & Tytgat J. 1999. Scorpion toxins specific for Na^+-channels. *European Journal of Biochemistry/FEBS* 264(2):287–300.

Riddle BR & Honeycutt RL. 1990. Historical biogeography in North American arid regions: an approach using mitochondrial-DNA phylogeny in grasshopper mice (Genus *Onychomys*). *Evolution* 44(1):1–15.

Rimsza ME, Zimmerman DR & Bergeson PS. 1980. Scorpion envenomation. *Pediatrics* 66(2):298–302.

Rowe, AH & Rowe MP. 2008. Physiological resistance of grasshopper mice (*Onychomys* spp.) to Arizona bark scorpion (*Centruroides exilicauda*) venom. *Toxicon* 52(5):597–605.

Rowe AH, Xiao Y, Rowe MP, Cummins TR & Zakon HH. 2013. Voltage-gated sodium channel in grasshopper mice defends against bark scorpion toxin. *Science* 342(6157):441–446.

Struyk AF, Markin VS, Francis D & Cannon SC. 2008. Gating pore currents in DIIS4 mutations of $Na_V1.4$ associated with periodic paralysis: saturation of ion flux and implications for disease pathogenesis. *The Journal of General Physiology* 132(4):447–464.

Stuart-Fox D, Moussalli A & Whiting MJ. 2008. Predator-specific camouflage in chameleons. *Biology Letters* 4(4):326–329.

Vicart S, Sternberg D, Fontaine B & Meola G. 2005. Human skeletal muscle sodium channelopathies. *Neurological Sciences* 26(4):194–202.

Vandendriessche T, Olamendi-Portugal T, Zamudio FZ, Possani LD, Tytgat J. 2010. Isolation and characterization of two novel scorpion toxins: The α-toxin-like CeII8, specific for Na_v1. 7 channels and the classical anti-mammalian CeII9, specific for Na_v1. 4 channels. *Toxicon* 56(4):613-23.

Voss RS & Jansa SA. 2012. Snake-venom resistance as a mammalian trophic adaptation: lessons from didelphid marsupials. *Biological Reviews* 87(4):822–837.

Watt DD. 1990. Neurotoxins in venom from the North American scorpion *Centruroides sculpturatus* Ewing. *Abstracts of Papers of the American Chemical Society* 200:80.

Webb JR. 2007. Slow inactivation of sodium channels: structural clues and disease associations. *Doctoral Dissertation presented to The University of Texas Southwestern Medical Center*. 155 pages.

Wingerd JS, Mozar CA, Ussing CA Murali SS, Chin YK, *et al*. 2017. The tarantula toxin β/δ-TRTX- Pre1a highlights the importance of the S1-S2 voltage-sensor region for sodium channel subtype selectivity. *Scientific Reports* 7(1):974.

Winters JJ & Isom LL. 2016. Developmental and regulatory functions of Na^+ channel Non-pore-forming & beta subunits. *Current Topics in Membranes* 78:315-351.

Wood JN & Baker M. 2001. Voltage-gated sodium channels. *Current Opinion in Pharmacology* 1(1):17–21.

Wood JN, Boorman JP, Okuse K & Baker MD. 2004. Voltage-gated sodium channels and pain pathways. *Journal of Neurobiology* 61(1):55–71.

Yu FH & Catterall WA. 2003. Overview of the voltage-gated sodium channel family. *Genome Biology* 4(3), p.207.

Zhang S, Gao B & Zhu S. 2015. Target-driven evolution of scorpion toxins. *Scientific Reports* 5:14973.

CHAPTER 1: Molecular mechanisms of resistance to paralytic and lethal scorpion (Arizona bark scorpion, *Centruroides sculpturatus*) toxins in a scorpion predator (southern grasshopper mice, *Onychomys torridus*)

INTRODUCTION

Voltage-gated sodium (Na_v) ion channels are complex transmembrane proteins that regulate the activity of excitable cells, such as neurons and myocytes. Na_v channels underlie the rising phase of the action potential, and, thus, are essential for neuronal signal transmission and muscle contraction (Hodgkin & Huxley 1952; Denac *et al. 2000)*. Because these Na_v channels are critical for survival, their structure and function is highly conserved across diverse animal taxa (Goldin 2002; Kruger & Isom 2016). Consequently, Na_v channels are ideal targets of naturally occurring predatory and anti-predatory chemical weapons that aim to disrupt neuromuscular activity.

Offensive and defensive neurotoxic weapons specific to Na_v channels have arisen independently in multiple taxa across the phylogeny of life (Mebs 2001; Al-Sabi *et al.* 2006; Clement *et al.* 2007; Vandendriessche *et al.* 2008; Watt & Simard 2008; Klint *et al.* 2012; Yang *et al.* 2013; Han *et al.* 2017; Prashanth *et al.* 2017; Drukewitz *et al.* 2018; Prentis *et al.* 2018). Among species that produce chemical weapons, scorpions are particularly intriguing. Compared to species like puffer fishes (Indumathi & Khora 2017), newts (Hanifin *et al.* 1999) and blue ringed octopus (Sheumack *et al.* 1978; Williams *et al.* 2011), which harbor the symbiotic bacteria that produce a single neurotoxic compound (tetrodotoxin, TTX), or poison frogs that assimilate toxic chemicals from their diet (Saporito *et al.* 2011) – scorpions evolved venom glands,

which express a family of genes that encode complex mixtures of toxins (low-molecular-weight peptides, small molecules) selective for both predator and prey. Moreover, scorpions have evolved a specialized delivery system, hypodermic needle-like stingers that inject toxins into potential enemies (predators, prey, competitors) (Gwee *et al.* 2002; Watt & Simard 2008).

Most species of scorpions that produce lethal neurotoxins belong to the family Buthidea (~500 species) (Possani *et al.* 1999). One desert-dwelling Buthid species, *Centruroides sculpturatus* (Arizona bark scorpion), produces painful and lethal neurotoxic venom as a defense against potential mammalian predators (van der Meijden *et al.* 2017). *C. sculpturatus* venom is a cocktail of peptides that selectively disrupt the function of Na_v channels (Possani *et al.* 1999; Possani *et al.* 2000; Bosmans & Tytgat 2007; Escalona & Possani 2013;), causing intense pain, paralysis of the diaphragm muscles, and fatal asphyxiation in small mammals (including human infants) (Patterson 1960; Cahalan 1975; Ismail 1995; LoVecchio & McBride 2003; Valdez-Cruz *et al.* 2004; Webber & Graham 2013).

Despite being chemically protected, these deadly scorpions are regularly preyed upon by carnivorous desert rodents called grasshopper mice (*Onychomys torridus*) (Rowe & Rowe 2006). Staged behavioral trials between grasshopper mice and *C. sculpturatus* show that the mice get stung numerous times during a hunt, but experience little discomfort, and are seldom deterred by the scorpion (unlike venom-sensitive house mice, *Mus musculus*) (Rowe & Rowe 2008). This apparent physiological resistance to pain and muscle paralysis seen in grasshopper mice is generally understood to be an adaptation to foraging in habitats where *C. sculpturatus*

scorpions are an abundant resource that are unavailable to venom-susceptible competitors. Prior work has shown that one Na_v channel isoform involved in the pain pathway, $Na_v1.8$, has acquired modifications in grasshopper mice that make the channel bind venom peptides and block pain signals (Rowe *et al.* 2013). While this work revealed the molecular mechanism underlying resistance to pain-inducing toxins in bark scorpion venom, *how* these carnivorous mice avoid muscle paralysis and death is not yet understood. To understand the molecular basis of resistance to the lethal components of *C. sculpturatus* venom, I investigated the role of $Na_v1.4$. I chose to study $Na_v1.4$ because it is the only Na_v channel paralog expressed in myocytes (muscle cells) (Goldin *et al.* 2000), and it is a known target of other *Centruroides* venoms in sensitive mammals (namely rats and humans).

Centruroides toxins manipulate the gating kinetics of sensitive Na_v channels (Catterall *et al.* 2007). The gating mechanism and function of a Na_v channel (Figure 1) is closely coupled to its structure. A typical mammalian Na_v channel is made of a large pore forming alpha subunit (~260 kDa) and two smaller accessory beta subunits (32-36 kDa) (Namadurai *et al*. 2015, Isom L. 2014). The Na_v alpha subunit has 4 homologous domains (DI – DIV) and 6 transmembrane segments (S1-S6) per domain (Figure 1) (Marban *et al.* 1998). In the three-dimensional structure, the extracellular, reentrant loops that connect S5 to S6 of each domain come together to line the channel pore (Guy & Seetharamulu 1986). The S4 segments of DI- DIV are called the voltage sensors because they contain positively charged amino acid residues that move outward in response to changes in membrane potential (Catterall 1986). This upward and outward movement of the voltage sensors causes a conformational change that

opens the Na_v channel pore (Catterall 2000; Catterall 2010). Activated channels remain selectively conductive to Na^+ ions for a few milliseconds, after which the hinged lid mechanism of the inactivation gate closes to block further influx of ions – the channel is inactivated. The intracellular loop connecting DIII to DIV forms the hinged inactivation lid of the channel, and its movement is linked to the voltage sensor of DIV, such that complete outward movement of the DIV voltage sensor segment pulls the hinged lid of the inactivation gate, blocking the pore on the intracellular end (Armstrong 1981; Stühmer *et al.* 1989; Goldin 2003). According to some studies, the sequential movement of DI-DIII voltage sensors may be sufficient for channel activation, while the primary function of the DIV voltage sensor is likely related to the movement of the inactivation gate (Campos *et al.* 2004; Chanda & Bezanilla 2002; Horn *et al.* 2000; Silva & Goldstein 2013; Bosmans *et al*. 2008).

C. sculpturatus toxins that target Na_v channels are peptides, typically ranging from 60 to 75 amino acids long (Possani *et al.* 1999; Gordon *et al.* 1998). The peptides are classified as either α or β toxins (designated α and β to distinguish from alpha and beta Na_v channel subunits) based on their binding sites and effects on the channels (Couraud *et al.*1982; Gordon *et al.* 1998). The α scorpion toxins in the venom of some *Centruroides* species primarily bind to the extracellular loop connecting S3-S4 segments in DIV (Tejedor & Catterall 1988; Thomsen & Catterall 1989), preventing the movement of the DIV voltage sensors and, consequently, the inactivation gate (see structure-function relationship above) (Kirsch *et al.* 1989; Rogers *et al*. 1996; Campos *et al.* 2008). Therefore, α-toxin bound channels activate normally, but experience delayed inactivation. On the other hand, β toxins bind to the extracellular loop connecting S3-S4

segments of the channel's DII, and open the channel prematurely (i.e. at more negative membrane potentials than physiologically normal) (Possani *et al.* 1999; Cestèle & Catterall 2000; Mantegazza & Cestèle 2005; Escalona & Possani 2013). Overall, scorpion-venom bound Na_v channels activate prematurely and remain active longer than necessary for normal physiological processes. In the pain pathway, these effects lead to anomalous and prolonged pain signals. However, in muscles, scorpion toxins induce unwarranted muscle contractions and twitches that can paralyze the diaphragm, causing respiratory failure (Rimsza *et al.* 1980).

In this study, I investigated the effects of venom from *C. sculpturatus* on native grasshopper mouse muscle $Na_v1.4$ channels expressed in *Xenopus* oocytes. My study is the first to demonstrate the effects of whole *Centruroides* venom on mammalian $Na_v1.4$ channels expressed in heterologous cells. I show that grasshopper mouse $Na_v1.4$ channels are less sensitive to *C. sculpturatus* venom than those of rats and house mice (control animals that die from *C. sculpturatus* envenomation) Further, I identify the specific structural modifications in the grasshopper mouse $Na_v1.4$ protein that are responsible for reducing the channel's sensitivity to certain effects of *C. sculpturatus* toxins. Remarkably, $Na_v1.4$ channels employ a mechanism of resistance that is significantly different from that seen in the grasshopper mouse $Na_v1.8$ channels of the pain pathway. My results suggest that these functionally conserved Na_v channels can evolve several structural modifications in response to multifarious selection imposed by venom, without significant adverse effects to overall physiology.

MATERIALS AND METHODS

In mammals, the $Na_v1.4$ protein is encoded by the *Scn4a* gene. To investigate the role of $Na_v1.4$ channels in muscle resistance to *C. sculpturatus* venom, I sequenced the complete coding region of the grasshopper mouse *Scn4a*. First, I extracted mRNA from the muscle tissue of three different grasshopper mouse species, and then used PCR and cloning to amplify the *Scn4a* gene for sequencing.

RNA isolation and cDNA synthesis

For RNA extraction from skeletal muscle of *Onychomys torridus* (3 animals), *O. arenicola* (2 animals), *O. leucogaster* (1 animal), and CD-1 *Mus musculus*, I used TRIzol (Ambion) and followed the manufacturer's protocol. I converted mRNA to cDNA (complementary DNA) using SuperScript III First Strand Synthesis Reverse Transcriptase PCR kit (Invitrogen). A gene specific reverse transcription primer (6327RT), along with Oligo $d(T)_{20}$ was used to prime the reverse transcription reaction for cDNA synthesis.

PCR and cloning for sequencing

I downloaded the coding region of *Scn4a* from multiple closely related species from the NCBI database and aligned them using the MUSCLE algorithm in Geneious (version 7.1.5). Based on the alignment, I designed degenerate primers to amplify the grasshopper mouse and house mouse *Scn4a* gene in two overlapping pieces using New England Biolabs' (NEB) Q5 DNA polymerase. Primer design and PCR conditions were determined based on manufacturer's guidelines. I then ran the amplified fragments

on a 0.8% w/v agarose gel, excised the appropriate sized bands, and purified the DNA using Wizard SV Gel and PCR Clean-Up System (Promega). These purified PCR products were sequenced at GENEWIZ.

Models of molecular evolution

To identify amino acids under positive selection in the *Onychomys* lineage, I used likelihood based models of molecular evolution in Codeml of PAML. I selected the branch-site model of Codeml because I was looking for episodic adaptive evolution on a few amino acids in a specific lineage (Zhang *et al*. 2005). I tested for an association between venom resistance in the *Onychomys* lineage and signatures of selection acting on the *Scn4a* gene. CODEML calculates a ratio of non-synonymous to synonymous substitutions, omega (ω), as a proxy for selection. When a protein is under negative or purifying selection, $\omega < 1$, whereas $\omega > 1$ indicates positive selection for structural/functional changes to the protein. When $\omega = 1$, neither substitution type is favored, and the protein is likely evolving under neutral pressures.

Based on species relationships from published studies (Fabre *et al.* 2012; Nyakatura & Bininda-Emonds 2012; Springer *et al.* 2012), I created a species phylogenetic tree, and manually converted it to Newick format. I added the *Scn4a* sequences of all *Onychomys* species to the previously generated multi-species alignment of *Scn4a* orthologous sequences (see PCR and cloning section) and exported the alignment to PHYLIP format supported by PAML. I set the *Onychomys* lineage as the "foreground" branch in which ω values could be estimated at > 1, whereas all other branches were "background" in which ω ranged from 0-1. Using the

likelihood ratio test, I compared this model to a null model that did not allow for positively selected sites and had a fixed ω of 1 in the "foreground" lineages.

Electrophysiology

Do the structural modifications in grasshopper mouse $Na_v1.4$ channels impart resistance to *C. sculpturatus* venom? To answer this question, I expressed the grasshopper mouse $Na_v1.4$ protein in *Xenopus laevis* oocytes, and then used the two-electrode voltage-clamp technique to evaluate the effects of *C. sculpturatus* venom on the voltage and time-dependent properties of channels. During these experiments, the $Na_v1.4$ channels of two sensitive mammals, rat (*Rattus norvegicus*) and house mouse (*Mus musculus*), served as controls. I limited comparisons to the $Na_v1.4$ alpha subunit by co-expressing the alpha subunit of all species with rat beta 1 and beta 2 accessory subunits.

PCR and cloning for expression in *Xenopus laevis* oocytes

For cloning of the *Scn4a* gene, I designed new primers (specific to grasshopper mouse and house mouse *Scn4a* coding regions) to amplify the full-length gene in one piece. Then I sub-cloned the purified PCR products into the p-GEM-t-easy vector following the manufacturer's protocol. Clones were checked by sequencing (GENEWIZ), and clones with the correct insert sequence were selected for further processing. Since neither the grasshopper mouse nor house mouse *Scn4a* sequence contained EcoRI restriction sites, I used this enzyme to excise the full gene from the p-GEM-t-easy vector for cloning into pcDNA3.1+ expression vector (contains the T7

promoter for *in vitro* RNA synthesis). I similarly digested pcDNA3.1+ with EcoRI and calf intestinal phosphatase (CIP), and ligated the sticky ends overnight using NEB's DNA ligase (manufacturer's protocol with 3:1 ratio of insert: vector). I transformed the ligated plasmid constructs into MAX Efficiency™ Stbl2™ competent cells (Invitrogen) and modified the manufacturer's protocol for sodium channel cloning according to Feldman & Lossin (2014). At each stage, I extracted plasmid DNA using either PureLink™ HiPure Plasmid Miniprep Kit or PureLink™ HiPure Plasmid Maxiprep Kit (ThermoFisher Scientific) depending on desired amount of DNA. I preserved all colony cultures (prior to plasmid preparation) in 50% glycerol and stored them at -80^{o}C for future use. Once cloned, I re-sequenced the entire coding region of *Scn4a* gene to confirm orientation and base identity.

Site directed mutagenesis

I created four different mutagenic constructs from the wildtype grasshopper mouse *Scn4a* clone: a) Triple mutant in which the isoleucine at position 333 (I333) and glutamic acid at position 334 (E334) were changed to glutamic acid E333 and glycine G334; similarly, tyrosine Y1182, lysine K1133 were changed to serine S1182, glutamic acid E1183, and the C-terminal insert, unique to grasshopper mice, was removed. b) Di mutant in which only isoleucine I333, glutamic acid E134 were changed to glutamic acid E333, glycine G134. c) Diii in which only tyrosine Y1182, lysine K1133 were changed to serine S1182, glutamic acid E1183. e) NoC mutant in which the C-terminal insert was removed from the wildtype grasshopper mouse construct. I used the NEB Q5® Site-Directed Mutagenesis Kit for all mutagenesis reactions and selected the primers and

cycle conditions according to manufacturer's guidelines (http://nebasechanger.neb.com/). All mutagenized constructs were completely re-sequenced at GENEWIZ to ensure the lack of unwanted mutations.

Expressing *Scn4a* in *Xenopus laevis* oocytes

I purchased *Xenopus laevis* oocytes from Xenopus1 (MI), and manually removed the follicles surrounding the oocytes using forceps. For incubating oocytes overnight, I used ND-96 culture media containing 1.8 mM calcium chloride (CaCl2), 2 mM potassium chloride (KCl), 1mM magnesium chloride (MgCl2), 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2.5 mM sodium pyruvate ($C_3H_3NaO_3$) and 0.5 mM theophylline. I also supplemented the culture solution with 100 mg/L gentamicin and adjusted to a pH of 7.5 with 2N NaOH.

To synthesize capped mRNA (cRNA) from all plasmid constructs, I used the mMESSAGE mMACHINE™ T7 Transcription Kit (Thermo-Fisher Scientific) following NotI linearization of the plasmid at the 3' end of the gene. Post-linearization, I used phase separation with phenol-chloroform to clean DNA of RNases before cRNA synthesis, and then quantified the synthesized cRNA with a nanodrop spectrophotometer (Thermo-Fisher Scientific). Because beta subunits are important modulators of alpha subunit activity, prior to injection of cRNA into *Xenopus* oocytes for expression, I mixed the alpha subunit cRNA of *Scn4a* genes from house mouse, grasshopper mouse and rat (gift from Dr. G.K.Wang) with rat *Scn1b* and rat *Scn2b* cRNA (coding for beta1 and beta2 accessory proteins, gift from Dr. G.K.Wang) in a 1:1:1 ratio. Injected oocytes remained at 16°C until recording, for a maximum of 4 days.

Recording

Borosilicate glass electrodes (heat = 729, Pull = 100, Vel = 50, Time = 50, Sutter Instruments) filled with 3M KCl served as the current (resistance < 1 MΩ) and voltage electrodes (resistance < 2 MΩ). I placed oocytes in 450 µl of filter sterilized recording solution (ND96 supplemented with 2 mM KCl, 1.8mM CaCal2, 1mM MgCl2 and 10mM HEPES and adjusted pH to 7.5 with 2N NaOH). I custom built the recording chambers from disposable petri dishes with hot-glue damns connected by salt bridges. Each chamber was used only once. My recording amplifier and digitizer for oocyte clamp were OC725C (Warner Instruments) and Digidata 1200A (Axon Instruments) with pCLAMP10.6 software (Axon instruments). Leak currents were subtracted using P/4 technique.

C. sculpturatus venom hydration and dilution

I hydrated lyophilized crude *C. sculpturatus* venom (Rowe *et al.* 2013) to 40 µg/µl using sterile water, and stored aliquots at -80^{o}C. I pre-washed venom tubes with 1.5 µg/µl of bovine serum albumin (BSA) to prevent venom from sticking to tube walls. To maintain osmolality of recording solution, I diluted venom in recording solution and BSA to a concentration of 1.5 µg/µl prior to use. Then I pipetted 50 µl of 1 µg/µl venom (final concentration of 0.15 µg/µl) directly into the recording chambers (final volume of 500 µl) to test the effects of venom on voltage and time dependent properties of Na_v channels.

Protocols, data extraction and analysis

I used Axon™ pCLAMP™ 10 Electrophysiology Data Acquisition & Analysis Software (Axon Instruments) to visualized current traces, Clampfit to extract data from current traces and R Studio for processing and statistical analysis. The electrophysiology protocols and statistical analyses are as follows:

1) Na_v channel activation: β toxins in *Centruroides* venom bind to the S3-S4 extracellular loop of DII causing two different effects: 1) a hyperpolarized shift in the voltage dependence of channel activation, and 2) an overall decrease in peak Na^+ current amplitude. To test these β effects of *C. sculpturatus* venom on *Xenopus* oocytes expressing the $Na_v1.4$ channels, I measured the voltage dependence of activation. I depolarized *Xenopus* oocytes expressing either rat, house mouse or grasshopper mouse $Na_v1.4$ channels to +10 mV from a holding potential of -100 mV in a series of 5 mV steps. Before each test potential, the oocytes were given a pre-pulse of +10 mV to induce brief channel activation for β toxin binding. At each potential I extracted peak current using Clampfit software (Axon Instruments) and created a csv file for further analysis in R Studio. To calculate normalized conductance from current-voltage curves, I used the following equation $G_{Na} = I_{max}/V\text{-}V_{Na,}$ where I_{max} is the peak current, V is the depolarized conditioning potential and V_{Na} is the reversal potential. V_{Na} was estimated for individual oocytes using the equation $I = [1+ \exp(-0.03937 * z^* (V\text{-}V_{50}))]^{-1} * g^* (V – V_{Na})$, where z is the gating charge, g is a factor related to the number of channels contributing to macroscopic current, V is the voltage and V_{50} is the voltage of half-maximal activation (as in Smith & Goldin 1998). The numeric value of conductance is directly related to the number of "active" $Na_v1.4$ channels. I fit all conductance-voltage

plots to a single or sum of two Boltzmann equations: $P/(1 + \exp((V_{50a}-V)/k1)) + ((1-P)/(1 + \exp((V_{50a}-V)/k2)$, where P is the proportion of channels, and k1 and k2 are slopes. For single Boltzmann fits, P = 1. To statistically compare conductance values between different species or channel types at one voltage step (-20mV), I used a two-way repeated measures ANOVA design with species, venom and their interaction as factors.

To compare reduction in peak Na^+ current post venom application, I plotted peak current across the range of membrane potentials and used a three-way repeated measures ANOVA in the nlme function of R Studio software package (factors: voltage, venom, species) to test for significant differences.

2) Na^+ current decay constant (tau): α toxins in *Centruroides* venom bind to the S3-S4 segment of DIV and hold the inactivation gate longer than normal. This α toxin-induced impairment of the inactivation mechanism can be measured as a change in the Na^+ current decay constant across a range of voltage steps. Using the voltage dependence of activation protocol described above, I fit the decay phase of each current trace to a single exponential equation and extracted the decay time constant parameter (tau). To ensure the capture of current from only "inactivating" channels, I used the distal part of the current trace (> 4 ms), and compared data obtained from different channels using a three-way repeated measures ANOVA (factors: species, venom, voltage).

3) Na^+ current decay slope: As another measure of inactivation kinetics, I obtained the slope parameters for current traces using the "statistics" function of the Clampfit software (https://www.moleculardevices.com/en/assets/tutorials-videos). In theory, the decay slope parameter yields the same information as tau, however, there

are two major differences. First, while single exponential equations are fit to the distal part of the decay trace (> 6 ms after depolarization), decay slopes are calculated based on peak Na^+ current. Second, decay slope values are standardized by peak Na^+ current for each given trace and are therefore not influenced by the amplitude of the current. I used a two-way repeated measure ANOVA (species, voltage) as factors to assess statistical significance for pre-venom data. To determine the extent to which venom alters the Na^+ current decay slope, I measured the proportional change in decay slope after venom treatment. The proportion was calculated as: [(slope of Na^+ current before venom – slope of Na^+ current after venom)]/ slope of Na^+ current before venom. Then, I used a two-way repeated measures ANOVA to assess statistical significance between species at different voltage steps.

4) Voltage dependence of steady-state inactivation: I used a standard two-pulse protocol to measure the voltage dependence of steady-state inactivation. I depolarized oocytes expressing either rat, house mouse or grasshopper mouse $Na_v1.4$ channels to +10 mV for 50 ms in 5 mV steps, from a holding potential of -100 mV. A test pulse to +10 mV enabled me to measure the fraction of non-fast-inactivated current over the range of voltages. I plotted normalized currents across voltage steps, and then fit those data to a single Boltzmann equation: $1/(1+ \exp((V-V_{50})/k))$.

5) Recovery from fast-inactivation: To measure $Na_v1.4$ recovery from fast inactivation, I applied a conditioning pulse to oocytes (+10 mV for 100 ms) to induce fast inactivation in channels, and then varied the recovery time periods (0-20 ms in 0.5 ms incremental steps). Current from the fraction of recovered channels could be measured with a test pulse to +10 mV. I did not fit the data to any standard equations because

there were no visible differences between channel types or pre and post venom treatments (overlapping ± 1SE).

RESULTS

Grasshopper mouse *Scn4a* encodes amino acid substitutions at conserved sites

Preliminary data showed that skeletal muscle from grasshopper mice was less sensitive to *C. sculpturatus* venom (A. Rowe, unpublished). The gene *Scn4a* encodes the alpha subunit $Na_v1.4$ expressed in muscle. A multi-species alignment comparing *Scn4a* sequences from three species of grasshopper mice (*Onychomys* spp.) with other rodents and non-rodent mammalian species revealed that grasshopper mouse *Scn4a* encodes amino acid substitutions and an insert at highly conserved sites in the channel protein (Figure 1A). Moreover, the results showed variation in *Scn4a* among the three grasshopper mouse species. A partial species alignment showed a negatively charged glutamate (E at position 333 in the protein) was shifted to an adjacent site in the DI pore module (Figure 1B). In the DIII pore module, a negatively charged glutamate at position 1183 was substituted with a positively charged lysine (K) in *O. torridus*, while the adjacent serine (S at position 1182) in all three species is substituted with tyrosine (Y) (Figure 1B). Finally, the C-terminus carried an insert that was unique to grasshopper mice. The insert in the three grasshopper mice species (Figure 1C) comprised seemingly random combinations of serines (S), alanines (A), prolines (P), valines (V) and leucines (L). *O. torridus* had the longest (48 amino acids) insert.

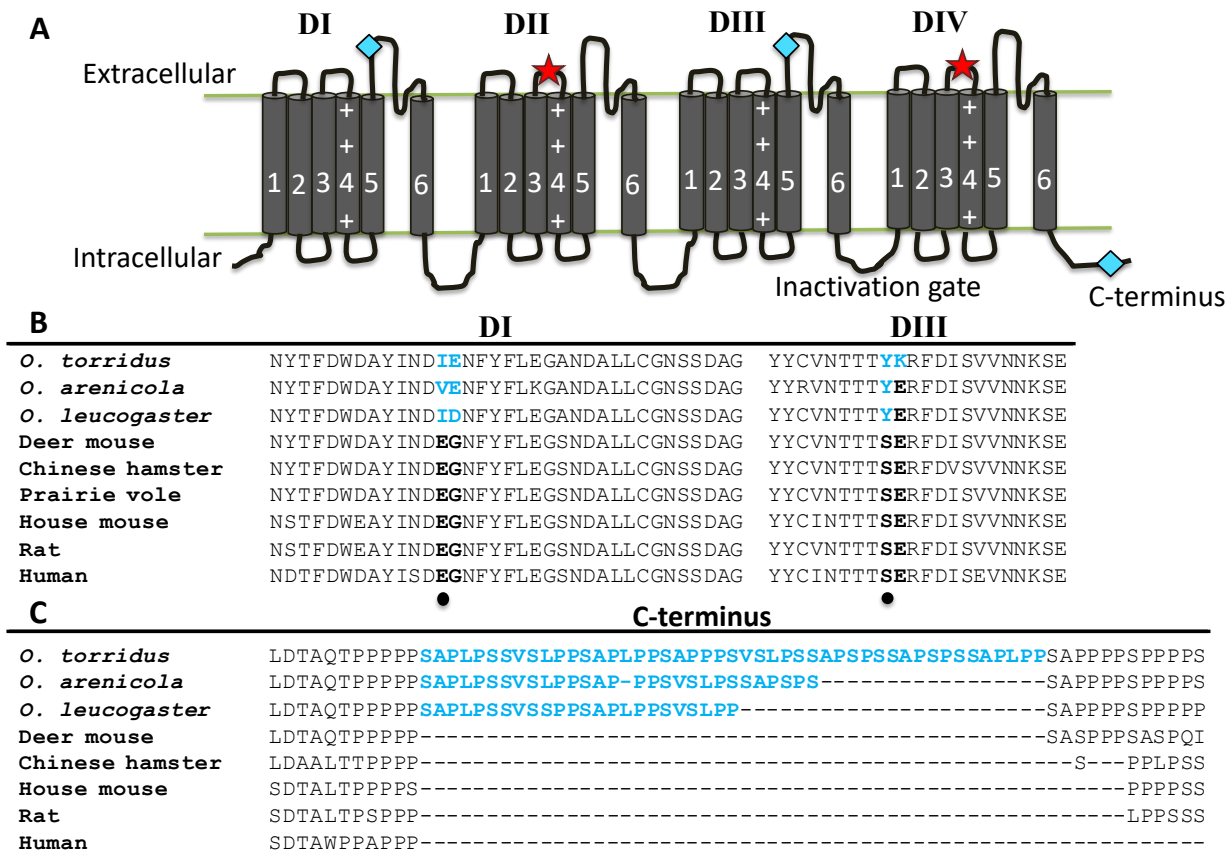

Figure 1: Na_v channel alpha subunit structure and sequence. A) Primary structure of a typical Na_v) channel alpha subunit. Blue diamonds represent location of unique substitutions seen in grasshopper mice, and red stars represent known *Centruroides* toxin binding sites. B) Partial multi-species amino acid sequence alignment of $Na_v1.4$. Amino acids of interest are shown in color. Dots below alignment indicate the amino acids under positive selection. Amino acid corresponding to the black dot in DI was under 97% positive selection in the *Onychomys* lineage, whereas the black dot in DIII shows amino acid under 67% positive selection. C) Insert in C-terminus unique to grasshopper mice is shown in color. Insert length varies among *Onychomys* species.

Models of molecular evolution to find amino acids under positive selection

Given that the *Scn4a* gene is highly conserved across taxa, I expected to see $\omega < 1$. However, many conserved proteins often experience episodic selection at specific active sites that may have subtle but significant effects on protein function (e.g. Jansa & Voss 2011). As expected, the branch-site model of CODEML found that the grasshopper mouse *Scn4a* gene had an $\omega << 1$, yet, seven amino acids (codons) in the *Onychomys* lineage were under positive selection (P = 0.012). Of these amino acids, E333I (transmembrane segment 5 of DI, 97% probability of positive selection) and S1182Y (transmembrane segment 5 of DIII, 67% probability of positive selection) are in extracellular loops physically close to known α and β toxin binding sites on the Na_v channel (Figure 1B).

Grasshopper mouse $Na_v1.4$ channels are less sensitive to *Centruroides* toxins

Effect of *C. sculpturatus* venom on activation

β toxin effect #1 (hyperpolarized shift in voltage dependence of activation) : The effect of β toxins on the voltage dependence of activation is demonstrated by the relationship between normalized conductance and voltage (Figure 2A-C). The voltage of half maximal activation (V_{50}) parameters derived from fitting the conductance-voltage relationship to a Boltzmann equation are shown in Table1. V_{50a} is the voltage of half maximal activation for channels that are unaffected by venom, whereas V_{50b} corresponds to the population of channels affected by venom. In the absence of venom there was a small but statistically significant difference between the V_{50a} of grasshopper mice vs. the control species (Figure 2A, Table 1). The V_{50a} of grasshopper mice (6.75

mV ± 0.15) was shifted 0.76 mV and 1.96 mV in the positive direction compared to the V_{50a} of house mice (z = -4.25; P << 0.01) and rat (z = -7.44; P << 0.01), respectively.

In the presence of venom, all species experienced premature, post-venom increases in conductance at voltages ranging from -55 to -10 mV (Figure 2B-C, Table 1). However, a greater number of rat channels were activated at hyperpolarized potentials compared to those of house mice or grasshopper mice. To make statistical comparisons, I chose one voltage step, -20 mV, at which the post-venom conductance was visually most different from pre-venom conductance. In the presence of venom, the normalized conductance in grasshopper mouse channels was significantly lower than in rat channels (t = 3.653; df = 2, 38; P << 0.01), but not house mouse channels (t = 0.68; df = 2, 38; P = 0.50). These data suggest that β toxins negatively shift the voltage dependence of activation of a larger number of rat channels compared to house mouse or grasshopper mouse channels.

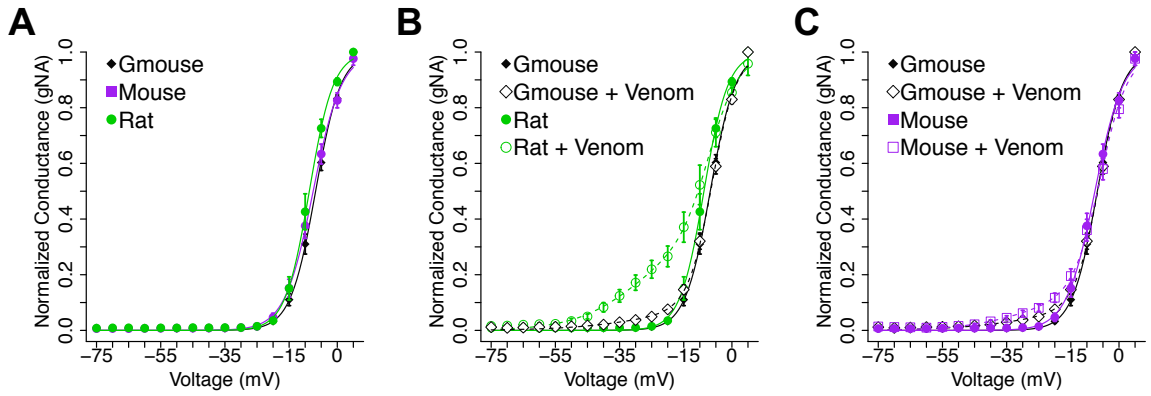

Figure 2: Effect of *C. sculpturatus* venom on the voltage dependence of activation of wildtype $Na_v1.4$ channels expressed in *Xenopus laevis* oocytes. **A)** Normalized conductance plotted as a function of membrane potential for grasshopper mouse (black), house mouse (purple) and rat (green) channels. **B)** Conductance-voltage

relationship pre (solid symbols) and post venom (open symbols) for grasshopper mouse (black) compared to rat $Na_v1.4$ (green). **C)** Conductance-voltage relationship in grasshopper mouse (black) $Na_v1.4$ channel compared to house mouse $Na_v1.4$ (purple) channels. All curves are fit the to a single (pre-venom) or double (post-venom) Boltzmann equation.

Species/$Na_v1.4$ construct	V_{50a}	K1	P	V_{50b}	K2	n
Gmouse	-6.75 ± 0.14	3.91 ± 0.12	1			15
Gmouse + venom	-6.15 ± 0.26	3.71 ± 0.18	0.91	-24.93 ± 5.82	12.70 ± 3.7	15
Mouse	-7.51 ± 0.21	4.41 ± 0.18	1			17
Mouse + venom	-5.75 ± 0.52	4.25 ± 0.38	0.87	-28.80 ± 10.20	10.25 ± 3.17	17
Rat	-8.71 ± 0.21	3.74 ± 0.18	1			9
Rat + venom	-7.07 ± 1.33	4.63 ± 1.14	0.68	-29.89 ± 13.44	9.95 ± 4.20	9

Table 1: Boltzmann parameters for normalized conductance vs. voltage relationships of wildtype grasshopper mouse, house mouse and rat $Na_v1.4$ channels. V_{50a} and V_{50b} are the voltages of half maximal activation for the proportion of channels affected and not affected by *C. sculpturatus* venom, respectively. K1 and K2 are the slope parameters, P is the proportion of channels unaffected by venom, and n is the number of oocytes recorded.

β toxin effect #2 (decrease in peak Na^+ current): To evaluate the effect of β toxins on the peak Na^+ current, I plotted normalized current as a function of voltage (Figure 3A-B). *C. sculpturatus* venom caused a significant reduction in peak Na^+ currents in all species ($P < 0.01$; df = 2, 76; t = 2.721) at voltages ranging from -5 mV to +55 mV. However, a three-way repeated measures ANOVA found post-venom peak currents were larger for grasshopper mice compared to rat channels (t = 2.086; df = 2, 76; P =

0.04), but there was no difference between grasshopper mice and house mice (t = 1.64; df = 2, 76; P = 0.105). Additionally, the difference in peak current (before vs. after venom) at +5 mV shows the greatest change occurred in rat $Na_v1.4$, whereas the smallest change occurred in grasshopper mice (Figure 3C). The data in Figure 3 illustrate that rat channels are most sensitive to the β-toxin effects of *C. sculpturatus* venom, house mice display intermediate sensitivity and grasshopper mice are the least sensitive.

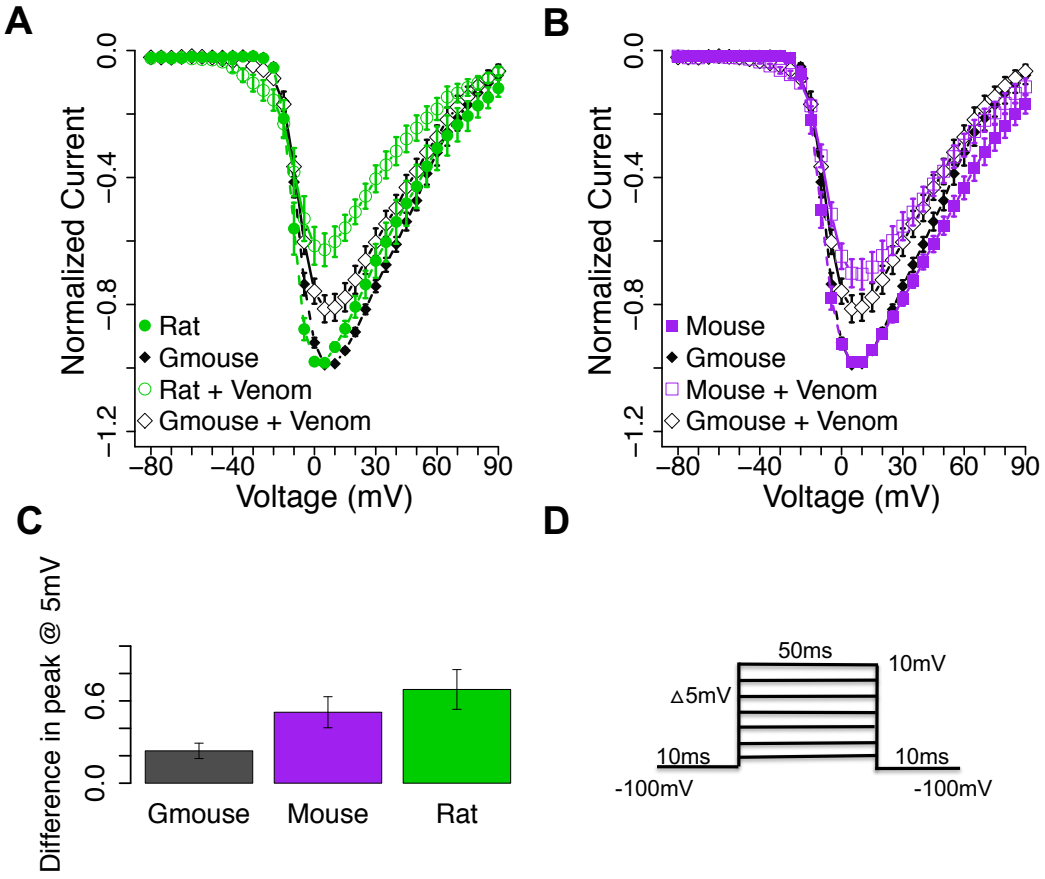

Figure 3: Effect of *C. sculpturatus* venom on voltage-current relationships for wildtype $Na_v1.4$ channels expressed in *Xenopus laevis* oocytes. All peak currents were normalized by pre-venom peak currents at each voltage step. A) Voltage-current

curves for rat (green) and grasshopper mouse (black) $Na_v1.4$ channels before (solid symbols) and after (open symbols) the application of 0.15 µg/µl of *C. sculpturatus* venom. B) Voltage-current curves for house mouse (purple solid) and grasshopper mouse (black solid) $Na_v1.4$ channels before and after (open symbols) the application venom. C) Difference between pre and post venom peak current observed at +5 mV for grasshopper mouse (grey), house mouse (purple) and rat (green). D) Illustration of activation protocol used to generate voltage current curves. All data represent mean ± 1SE.

Effect of *C. sculpturatus* venom on inactivation kinetics: To determine the α-toxin effect of *C. sculpturatus* venom on $Na_v1.4$, I applied a single depolarizing potential of 0 mV for 50 ms to oocytes expressing either grasshopper mouse or control $Na_v1.4$ channels, and then overlaid the current traces produced before and after the application of venom. In the absence of venom (solid lines), representative current traces from all three species demonstrated rapid inactivation of Na^+ currents (Figure 4A). In contrast, the application of *C. sculpturatus* venom (dashed lines) slowed the decay of Na^+ current in grasshopper mice, house mice and rats, suggesting α-toxin inhibition of the fast inactivation mechanism in all three species (Figure 4A).

I quantified α toxin effects on Na^+ current inactivation by measuring two different parameters: 1) time constant of Na^+ current decay (tau); and 2) slope of the Na^+ current decay.

Effect of α toxin on tau: When averaged across voltages ranging from -25 mV to +30 mV, there were no statistically significant baseline differences in the tau values of

grasshopper mice, house mice and rats (Figure 4B; F-value = 2.0739; df = 2, 38; P = 0.140). However, at certain voltages there were significant differences in the baseline tau values of the three species (F-value = 2.409; df = 22, 416; P << 0.01). For example, at -20 mV, the tau of grasshopper mouse channels was significantly higher than that of rat (t-value = -2.391; df = 416; P = 0.017), and marginally higher than house mice (t-value = -1.804; df = 416; P = 0.072). At -25 mV, the tau value for grasshopper mouse channels was significantly higher than that of house mouse channels (t-value = -4.230, df = 416; P < 0.01) but not rat channels (t-value = -0.527; df = 416; P = 0.598).

The application of venom significantly increased the Na^+ current tau values in all three species (Figure 4C and 4D; df = 1, 454; F-value = 58.364; P << 0.01). At -25 mV, the tau of grasshopper mouse channels was significantly higher than house mice channels (t-value = 3.866; df = 454; P << 0.01) but not rat channels (t- value = -0.421; df = 454; P = 0.6740). At all other voltages, venom had similar effects on the tau of grasshopper mice, house mice and rats.

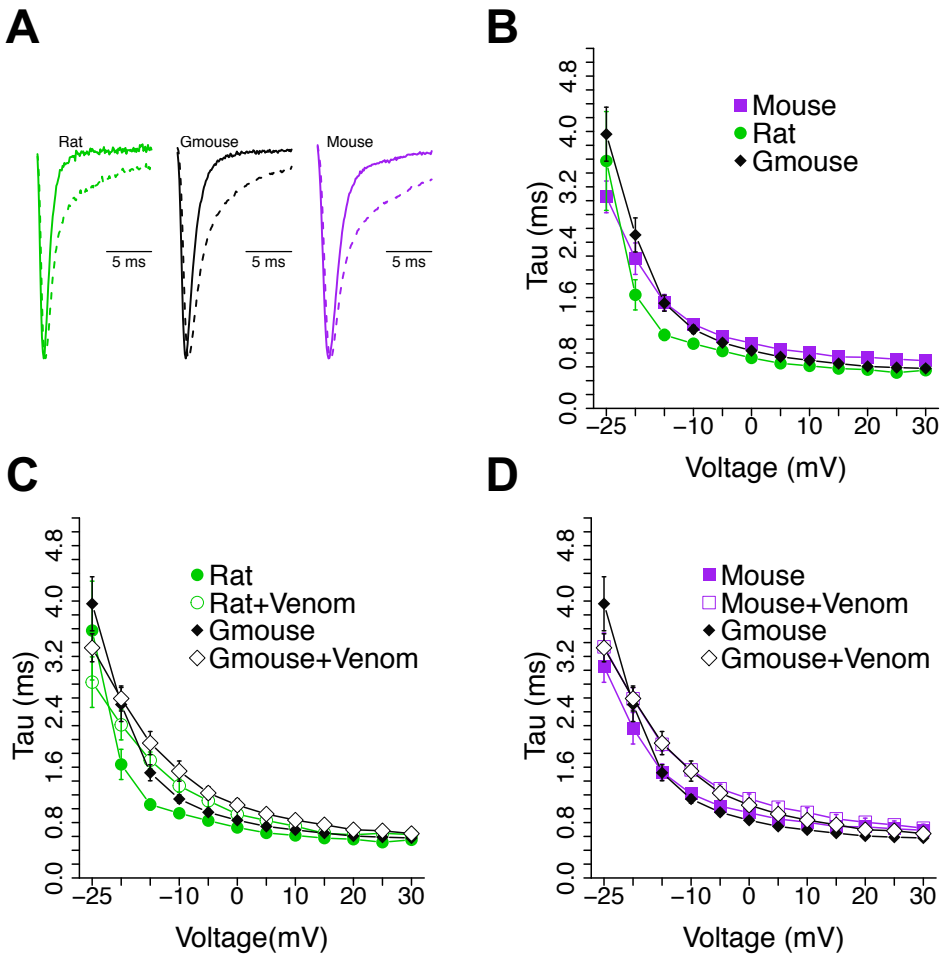

Figure 4: Effects of *C. sculpturatus* venom on inactivation kinetics of $Na_v1.4$ channels expressed in *Xenopus laevis* oocytes. A) Na^+ current traces generated from a single 0mV depolarizing pulse. Solid lines show pre-venom current for rat (green circles), grasshopper mouse (black diamonds), and house mouse (purple squares) channels, whereas dashed lines are post venom current traces. B) Time constant of Na^+ current inactivation (tau) in absence of venom for grasshopper mice (black), house mice (purple) and rats (green). Effect of venom on the time constant of inactivation of C) rat channels (green open circles) compared to grasshopper mouse (black open diamonds), and D) house mouse channels (purple open squares) compared to grasshopper mouse (black open diamonds).

Effect of α toxin on Na^+ current decay slope: I measured the slope of Na^+ current decay over a range of membrane potentials, before and after venom (Figure 4). In the absence of venom there were species-specific differences in current decay slopes at membrane potentials ranging from -15 to +10 mV (Figure 4A). A two-way repeated measures ANOVA revealed that the decay slope for grasshopper mouse channels was significantly shallower than that of rats ($df = 2, 37$; $t = 2.49$; $P = 0.017$), but not house mice ($t = 1.34$; $df = 2, 37$; $P = 0.19$) (Figure 4D). Shallower Na^+ current decay slopes and higher tau values produced by grasshopper mouse $Na_v1.4$ suggests that these channels experience delayed inactivation kinetics (compared to control channels) under baseline conditions.

In the presence of *C. sculpturatus* venom, the decay slopes of all species appeared less steep than baseline (Figure 5C, 5D). To determine the extent of change in the decay slope, I measured the proportion change in slope from baseline (Figure 5B). At voltages ranging from -10 mV to +30 mV, there was a significant species effect on proportion change in decay slope (F-value = 4.289; $df = 2, 37$; $P = 0.021$). The proportion change of decay slope from baseline for grasshopper mice was significantly lower than rats and house mice across the range of voltages. These results show that α toxins have a significantly smaller debilitating effect on the inactivation mechanism of grasshopper mouse $Na_v1.4$ channels compared to controls.

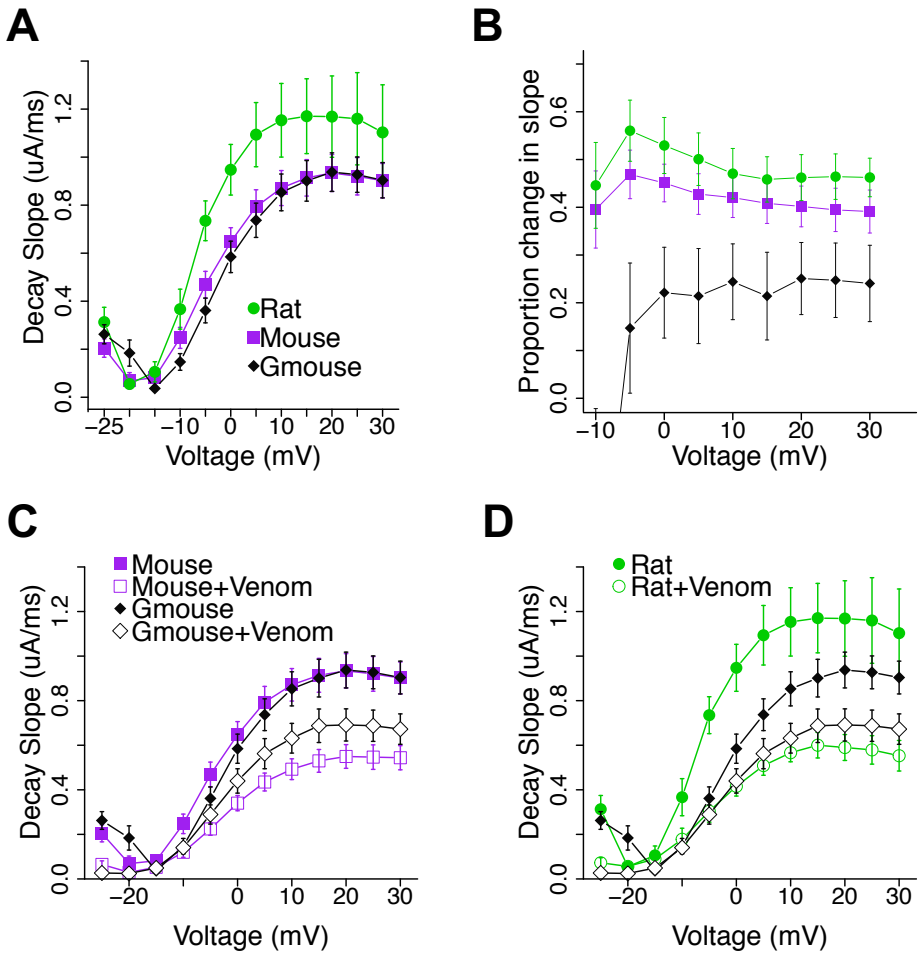

Figure 5: Effects of *C. sculpturatus* venom on the decay slope of $Na_v1.4$ current. A) Decay slope of Na^+ current plotted across voltage steps ranging from -25mV to +30mV for grasshopper mice (black) and house mice (purple) before (solid symbols) in the absence of venom. B) Proportion change in slope of Na^+ currents due to venom application, calculated as [(pre venom slope – post venom slope)/ pre venom slope]. C) Decay slope of Na^+ current plotted across voltage steps comparing grasshopper mouse to house mouse channels before (solid symbols) and after (open symbols) the application of venom. D) Decay slope of Na^+ current plotted across voltage steps comparing grasshopper mouse to rat channels before (solid symbols) and after (open symbols) the application of venom.

Steady-state inactivation and recovery: In addition to voltage dependence of activation and fast inactivation kinetics, I also measured the voltage dependence of steady-state inactivation and recovery from fast inactivation. I found no differences between the three species in their voltage dependence of steady-state inactivation or rate of recovery from fast inactivation either in the presence or absence of *C. sculpturatus* venom (data not shown).

Role of unique amino acid modifications in conferring resistance to *C. sculpturatus* venom

Models of molecular evolution found seven amino acids to be under positive selection in the *Onychomys* lineage. Of these, I333 (S5-SS1 loops of DI) and Y1182 (S5-SS1 loops of DIII) are located in extracellular loops that are in close proximity to α and β toxin binding sites on the Na_v channel. To test the role of DI and DIII amino acids substitutions in reducing the $Na_v1.4$ channel sensitivity to *C. sculpturatus* toxins, I changed two amino acids in each of these locations (i.e., DI: I333 to E333 and E334 to G334; DIII: Y1182 to S1182 and K1183 to E1183) to the presumed ancestral residue (as seen in house mice and rats). Further, the wildtype grasshopper mouse $Na_v1.4$ also carries a unique 48-amino acid insert in its C-terminus region. Because amino acids in the C-terminus play an important role in regulating the inactivation kinetics of Na_v channels, this insert may be important for reducing sensitivity to scorpion toxins. Therefore, I removed the 48-amino acid C-terminus insert from the previously mutated grasshopper mouse $Na_v1.4$ channel (referred to as the “triple mutant” channel). Other than the mutations I introduced into DI, DIII and C-terminus, the triple mutant channel

was identical to the wildtype grasshopper mouse $Na_v1.4$. Using previously described voltage-clamp technology and protocols, I tested the electrophysiological properties of this triple mutant channel and compared it to the wildtype grasshopper mouse $Na_v1.4$ channel.

Species/$Na_v1.4$ construct	V_{50a}	K1	P	V_{50b}	K2	n
Triple mutant	-10.53 ± 0.09	4.07 ± 0.08	1			87
Triple mutant venom	-12.14 ± 0.21	4.60 ± 0.19	0.94	-29.49 ± 3.61	17.33 ± 1.89	87
Gmouse	-8.93 ± 0.09	4.11 ± 0.07	1			86
Gmouse + venom	-10.14 ± 0.14	4.47 ± 0.13	0.96	-22.71 ± 4.61	15.33 ± 3.70	86

Table 2: Boltzmann parameters for normalized conductance vs. voltage relationships of wildtype grasshopper mouse and triple mutant $Na_v1.4$ channels. V_{50a} and V_{50b} are the voltages of half maximal activation for the proportion of channels affected and not affected by *C. sculpturatus* venom, respectively. K1 and K2 are the slope parameters, P is the proportion of channels unaffected by venom, n is the number of oocytes recorded.

β toxin effects # 1 (hyperpolarizing shift in voltage dependence of activation) on triple mutant channels: I plotted conductance-voltage curves for triple mutant channels and compared them to wildtype grasshopper mouse channels (Figure 6A). As before, I fit conductance-voltage curves to a Boltzmann equation and statistically compared the V_{50} parameters. In the absence of venom, I found a small but significant difference in the Boltzmann fit parameter, V_{50a} (Table 2), between the wildtype grasshopper mouse and triple mutant channels. On average, the V_{50a} of the triple mutant was shifted 1.6 mV in the negative direction compared to the wildtype

grasshopper mice channel (z = -13.68; P << 0.01). This hyperpolarizing shift in the V_{50a} of the mutant channel suggests that the amino acid modifications in grasshopper mouse $Na_v1.4$ channels are responsible for the baseline difference in V_{50a} between grasshopper mice and control species.

In the presence of venom, the difference between the V_{50b} parameters of grasshopper mice and the triple mutant was only marginally significant (z = -1.87; P = 0.06, Table 2). However, the conductance-voltage curve showed that the triple mutant had higher conductance than grasshopper mouse channels at voltages ranging from -40 to -10 mV. These results are shown in Figure 6B which are enlarged data from 6A to which the post-venom tends have been added. Indeed, the conductance of the triple mutant channels was significantly higher than grasshopper mouse channels at -20 mV in the presence of venom (t = 3.97; df = 1, 168; P << 0.01). These data suggest that triple mutant channels are more sensitive than grasshopper mouse channels to the effects of β toxins on the voltage dependence of channel activation.

β toxin effects # 2 (reduction in peak Na^+ current amplitude) on triple mutant channels: Next, I measured the β-toxins effects of *C. sculpturatus* venom on peak Na^+ current at depolarized membrane potentials (Figure 6C and 6D). I found that *C. sculpturatus* venom decreases peak Na^+ currents in both grasshopper mice and triple mutant channels (overlapping ± 1SEs). The difference between pre- and post-venom currents at +5 mV for grasshopper mice and triple mutants is shown in Figure 6D (t = -0.121; df = 1, 342; P = 0.904).

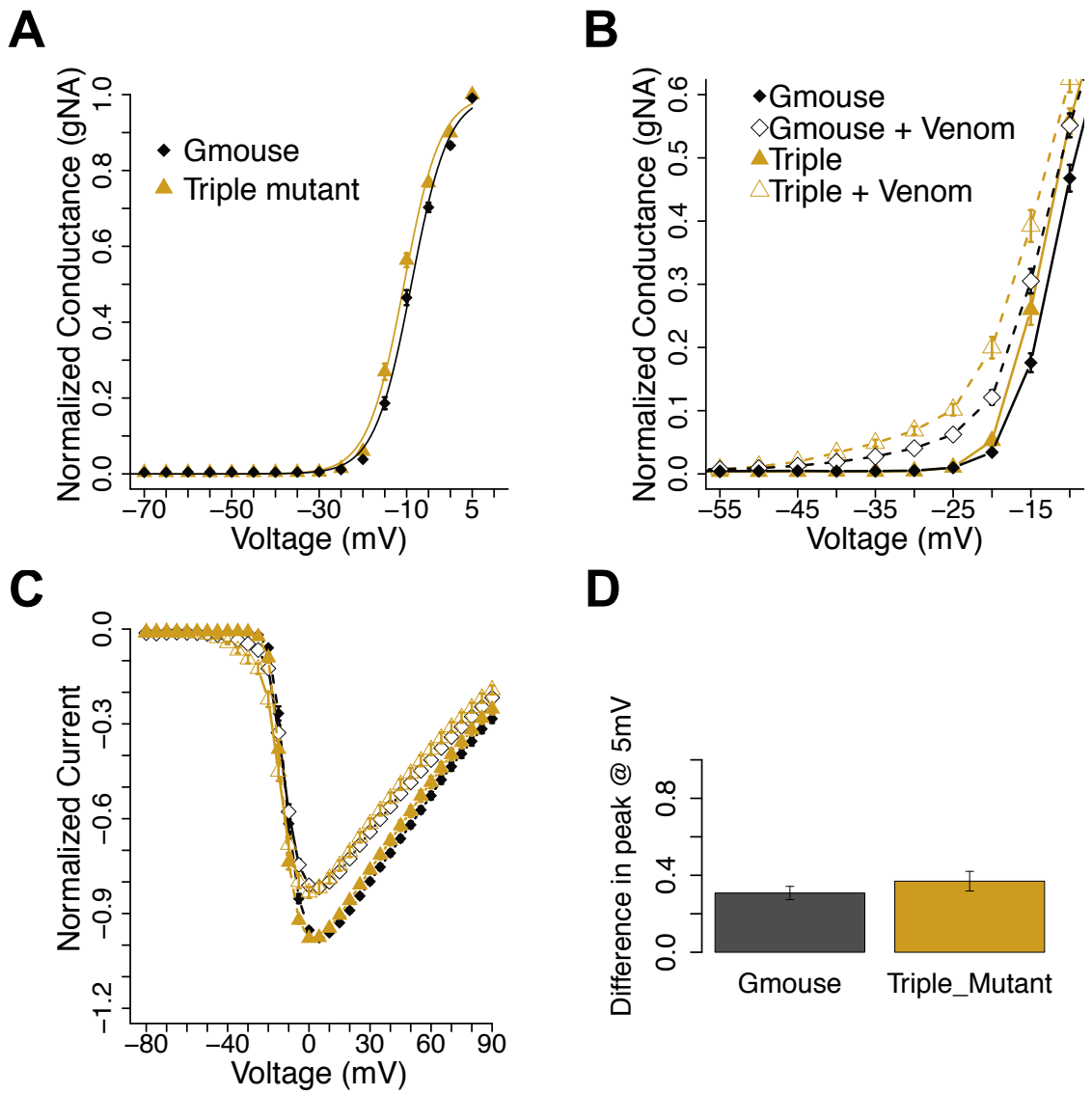

Figure 6: Effect of *C. sculpturatus* venom on the activation of triple mutant $Na_v1.4$ channels expressed in *Xenopus laevis* oocytes. A) Baseline conductance-voltage relationship of triple mutant channels (gold triangles) overlaid over wildtype grasshopper mouse channels (black diamonds). Data are fit to a single Boltzmann equation. B) Conductance-voltage relationship of triple mutant channels (gold triangles) compared to wildtype grasshopper mouse channels (black diamonds) in the absence (solid symbols) or presence (open symbols) of venom. Connecting lines do not represent fits. C)

Current-voltage relationship for triple mutant (gold triangles) and grasshopper mouse (black diamonds) channels. D) Difference between pre and post venom peak current observed at +5 mV for grasshopper mouse (black) and triple mutant channels (gold). Data are reported as mean ± 1SE.

Effect of α toxin on tau: To test the effects of α toxins on the triple mutant channel's inactivation kinetics, I measured tau for triple mutant and wildtype grasshopper mouse $Na_v1.4$ channels and found no difference either in the absence (F-value = 1.634; df = 11, 583; P = 0.086) or presence of venom (df = 11; 635; F = 0.163; P = 1.0; Figure 7A).

Effect of α toxin on Na^+-current decay slope: Finally, I measured the Na^+ current decay slope for grasshopper mouse and triple mutant channels (Figure 7B) and found no difference between the two types of channels either before or after venom application. Further, there was no difference in the proportion change in post-venom decay slope (Figure 7C, overlapping ± 1SE; df = 1, 12; F-value = 1.609; P = 0.2287).

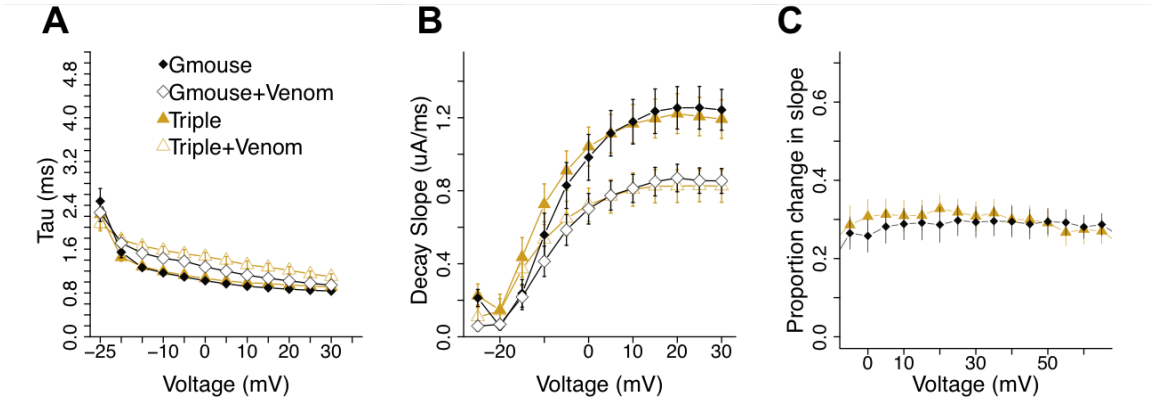

Figure 7: Effect of *C. sculpturatus* venom on the inactivation kinetics of triple mutant $Na_v1.4$ channels to measure α-toxin effects. A) Tau plotted across voltage

for triple mutant (gold triangles) and grasshopper mouse (black diamonds) channels before (solid symbols) and after (open symbols) venom application. B) Na^+ current decay slope plotted across voltage steps for triple mutant (gold) and wildtype grasshopper mouse channels (black) in the presence (open symbols) and absence (solid symbols) of venom. C) Proportion change in post-venom Na^+ current decay slope plotted across voltage steps for triple mutant (gold) and wildtype grasshopper mouse channels (black).

Relative contributions of DI, DIII and C-terminus insert towards reduced *C. sculpturatus* venom sensitivity

The electrophysiology recordings from the triple mutant construct suggested that structural modifications to grasshopper mice $Na_v1.4$ are involved in reducing sensitivity to *C. sculpturatus* venom β-toxin effects. To understand the relative contributions of the different regions (DI, DIII and the C-terminal insert) in reducing sensitivity to venom, I made three individual mutant channels: 1) Di: two amino acid residues I333 and E334 were changed to the presumed ancestral state (E333 and G334, respectively) in domain I; 2) Diii: two amino acid residues Y1182 S1182 and K1183 changed to the presumed ancestral type E1183 in domain II; 3) NoC: only the C-terminus insert was removed. This series of experiments focused on the electrophysiology property that varied between wildtype grasshopper mouse and triple mutant $Na_v1.4$ channels in the presence of venom – the voltage dependence of activation.

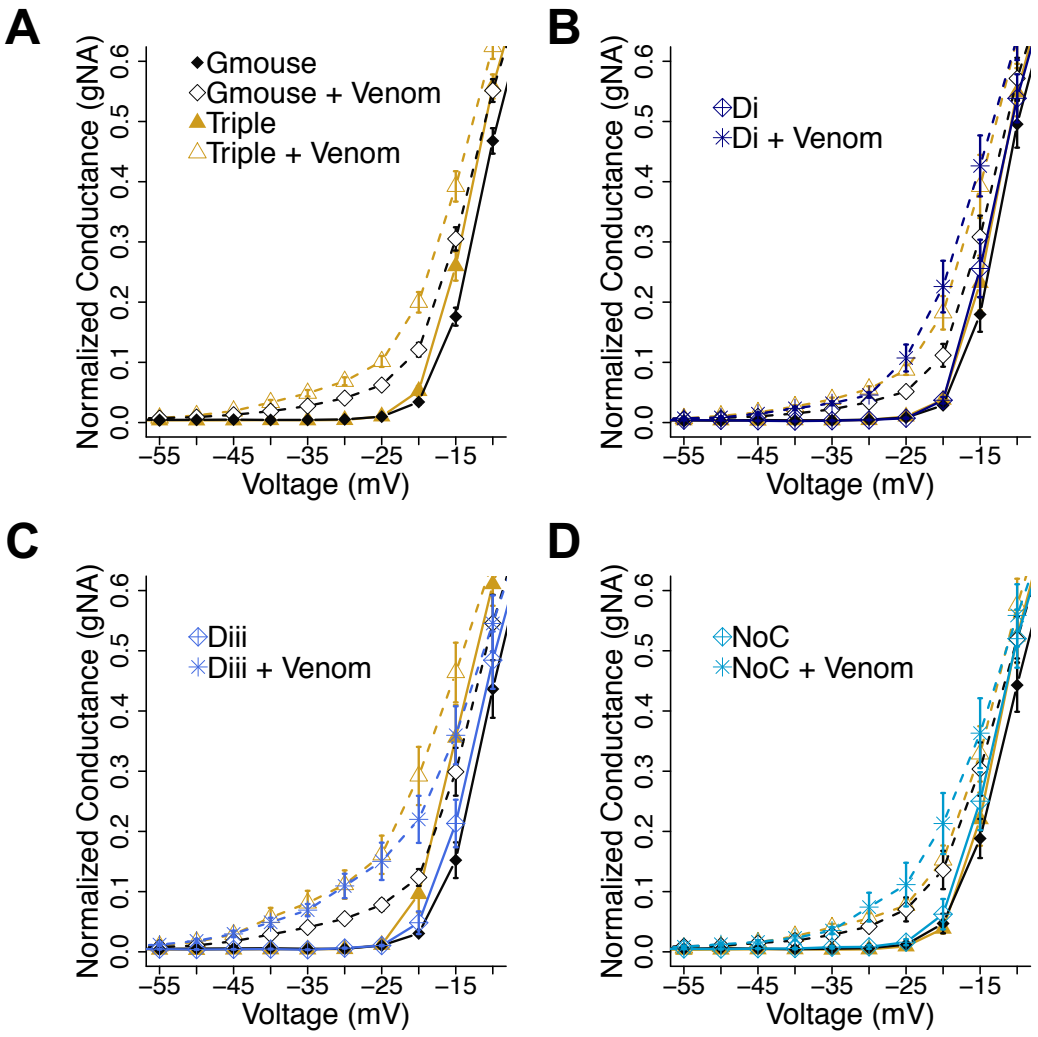

Figure 8: Effect of *C. sculpturatus* toxins on the voltage dependence of activation of Di, Diii and NoC mutant channels. Conductance-voltage relationships before (solid) and after (open symbols) venom for A) Triple mutant channels (gold triangles) and wildtype grasshopper mouse (black diamonds) $Na_v1.4$ channels; B) Grasshopper mouse, triple mutant and Di mutant channels (navy blue); C) Grasshopper mouse, triple mutant and Diii mutant channels (indigo); D) Grasshopper mouse, triple mutant and NoC mutant channels (sky blue). Electrophysiology and activation protocols are described previously. Data represent mean ± 1SE.

Experiment	Species/Nav1.4 construct	V_{50a}	K1	n
Di	Triple mutant	-10.27 ± 0.23	3.99 ± 0.2	20
	Gmouse	-9.13 ± 0.24	4.13 ± 0.20	19
	Di	-10.35 ± 0.16	3.92 ± 0.14	21
Diii	Triple mutant	-11.67 ± 0.21	4.44 ± 0.17	19
	Gmouse	-8.52 ± 0.29	3.99 ± 0.26	17
	Diii	-9.26 ± 0.28	4.32 ± 0.23	23
NoC	Triple mutant	-10.15 ± 0.20	3.66 ± 1.677	18
	Gmouse	-8.51 ± 0.29	4.50 ± 0.24	21
	NoC	-9.48 ± 0.3	4.72 ± 0.26	19

Table 3: Boltzmann parameters comparing normalized conductance vs. voltage relationships for wildtype grasshopper mouse, triple mutant and single mutant (Di, Diii, NoC) $Na_v1.4$ channels. V_{50a} is the voltage of half maximal activation, K1 is the slope parameter and n is the number of oocytes recorded. Only pre-venom data is shown.

Effect of DI mutations on sensitivity to *C. sculpturatus* venom: To test the sensitivity of the Di mutant to venom β toxin effects, I compared the voltage dependence of activation for the Di mutant to the wildtype $Na_v1.4$ before and after venom. In the absence of venom, I found baseline differences in the V_{50a} of Di mutants compared to grasshopper mice, but not triple mutant channels (Table 3). On average, the V_{50} of Di channels was shifted in the hyperpolarizing direction from grasshopper mouse channels by 1.22 mV ($z = 5.07$; $P << 0.01$). These data suggest that Di mutant channels have a higher baseline excitability than grasshopper mouse channels (but not triple mutant channels). In the presence of venom, there were no differences between V_{50b} values of the Di, triple mutant and wildtype $Na_v1.4$ channels. However, the conductance of Di mutant channels was significantly higher than that of wildtype

grasshopper mouse channels at -20 mV (t = 2.79; df = 2, 57; P < 0.01, Figure 8A), but did not differ from the triple mutant (t = -1.39; df = 2, 57; P = 0.17; Figure 8A and 8B). This suggests that the Di mutant is more sensitive to β toxin effects (similar to the triple mutant) than the wildtype grasshopper mouse $Na_v1.4$ construct.

Effect of DIII mutations on sensitivity to *C. sculpturatus* venom: To test the sensitivity of the Diii mutant to venom β-toxin effects, I compared the voltage dependence of activation for the Diii mutant to the wildtype $Na_v1.4$ before and after venom. In the absence of venom, I found baseline differences in the V_{50a} of Diii mutants compared to both grasshopper mice and triple mutant channels (Table 3); Diii had an intermediate V_{50a} value (Table 3). On average, the V_{50a} of Diii was lower than the grasshopper mouse channels by 0.77 mV (z = 2.79; P = 0.005) but higher than the triple mutant channels by 2.41 mV (z = -8.67; P << 0.01). These data suggest that Diii mutant channels have a higher baseline excitability than grasshopper mouse channels, and a lower baseline excitability than triple mutant channels. In the presence of venom, there were no significant differences between the V_{50b} values of Diii, triple mutant or grasshopper mouse channels. However, the Diii mutant channels had a marginally higher conductance than the wildtype channels at -20 mV (t = -1.81; df = 2, 56; P = 0.07, Figure 8C), but not the triple mutant channels (t = 1.16; df = 2, 56; P = 0.25). This suggests that a larger proportion of the Diii mutant channels are sensitive to β toxin effects than the wildtype grasshopper mouse $Na_v1.4$ channels.

Effect of C-terminal insert on sensitivity to *C. sculpturatus* venom: To test the sensitivity of NoC mutant to venom β-toxin effects, I compared the voltage dependence of activation of NoC mutant and the wildtype $Na_v1.4$ before and after

venom. I found baseline differences between the V_{50a} of NoC mutant and grasshopper mouse channels but not the triple mutant channels. On average, the V_{50a} of NoC channels was shifted in the hyperpolarizing direction from grasshopper mouse channels by -1.38 mV (Table 3; z= 4.75; P << 0.01). These data suggest that NoC mutant channels have a higher baseline excitability than grasshopper mouse channels, but not the triple mutant channels. In the presence of venom, there were no differences between the V_{50b} values of all species. Additionally, the Na^+ conductance for NoC mutants at -20 mV was only marginally different from grasshopper mice (t = -1.77; df = 2, 54; P = 0.08; Figure 8D), but there was no difference between NoC and triple mutant channels (t= 0.10; df = 2, 54; P = 0.32). This suggests that the NoC mutant has an intermediate level of sensitivity to β toxin effects compared to the wildtype and triple mutant $Na_v1.4$ channels.

DISCUSSION

Grasshopper mouse $Na_v1.4$ channels are less sensitive to the α- and β-toxin effects of *C. sculpturatus* venom

$Na_v1.4$ is the only Na_v channel paralog expressed on the surface of mammalian skeletal muscle cells (myocytes), where its function is crucial for muscle contraction (Goldin *et al.* 2000). Previous studies showed that $Na_v1.4$'s activation and inactivation (or gating) mechanisms are disrupted by the application of α and β toxins from *Centruroides elegans* and *C. vittatus* venoms (Vandendriessche *et al.* 2010; Rowe *et al*. 2011).Venom-induced modification of the $Na_v1.4$ gating mechanism hyperexcites the myocytes of sensitive mammals (e.g. house mice, rats, humans) (Bosmans & Tytgat

2007). Hyperexcitation leads to a block of membrane depolarization and loss of muscle contraction. Impaired contraction of the diaphragm can cause respiratory failure and death.

Grasshopper mice are resistant to *C. sculpturatus* venom, both natural stings and controlled injections (Rowe *et al.*, 2005 and 2008). Preliminary data showed grasshopper mouse muscle contraction was unaffected by *C. sculpturatus* venom, whereas house mouse muscles experienced a block in contraction (A. Rowe, unpublished). In this study, I tested the hypothesis that grasshopper mouse muscles are resistant to *C. sculpturatus* venom because they express structural modifications in $Na_v1.4$ that impart resistance to α- and β-toxin effects.

Centruroides β toxins contribute to membrane hyperexcitability by interacting with specific amino acids in Na_v domain II (DII) voltage sensors, causing a hyperpolarizing shift in the voltage dependence of channel activation (Cestèle *et al.* 1998; Cestèle & Catterall 2000; Mantegazza & Cestèle 2005). This β-toxin effect on activation makes channels more likely to open at resting membrane potentials. I tested the β-toxin effects of *C. sculpturatus* venom on the wildtype $Na_v1.4$ channels of grasshopper mice and compared their functional properties to the $Na_v1.4$ channels of house mice and rats (control species). As predicted, venom had a significantly smaller β-toxin effect on the voltage dependence of activation of grasshopper mouse $Na_v1.4$ channels compared to rat channels (Figure 2). Moreover, β toxins had a significantly smaller inhibitory effect on the peak current of grasshopper mouse channels compared to rat, but not house mouse channels. My results show that grasshopper mouse $Na_v1.4$ channels are less sensitive than rat channels to the β toxin effects of *C. sculpturatus* venom. However, house

mouse $Na_v1.4$ were also less sensitive to β-toxin effects compared to rats (Figure 2C). This might suggest that house mouse $Na_v1.4$ channels carry a different set of structural modifications, perhaps in less conserved regions of the channels, that impart reduced sensitivity to β toxins. Alternatively, house mice and grasshopper mice may share a subset of substitutions in other extracellular loops or less conserved regions (absent in rat channels) that confer reduced sensitivity to β toxins. Understanding the molecular mechanism underlying this reduced β toxins sensitivity in house mice compared to grasshopper mice may provide key insights into the evolvability of highly conserved and functionally important ion channel proteins.

Centruroides α toxins contribute to membrane hyperexcitability by targeting amino acids in the Na_v DIV voltage sensor, impairing the channel's fast inactivation mechanism (Catterall 1979; Bosmans & Tytgat 2007). By preventing the outward movement of the DIV voltage sensors, venom immobilizes the inactivation gate, and causes the channel to remain conductive for longer than physiologically normal (Catterall 1980; Kharrat *et al.* 1989; Campos *et al.* 2008; Wang *et al.* 2011). This effect manifests as a decrease in the slope of the Na^+ current decay; thus, α toxin-bound Na_v channels produce Na^+ currents that activate normally, but that do not inactivate within milliseconds, causing a shallow decay phase (Campos *et al.* 2008). I found that venom reduced the decay time constant (tau) similarly in grasshopper mice as well as the control species (Figure 4A). Only at -25 mV was the tau of grasshopper mouse $Na_v1.4$ channels significantly higher than house mouse channels in the presence of venom. However, the effect size of this difference was small (difference in effect size between grasshopper mouse vs. house mouse tau = 0.98 ± 0.25 ms) and is therefore unlikely to

represent a biologically meaningful difference. Although venom had similar effects on the tau values in all three species, I found that the baseline tau of grasshopper mouse $Na_v1.4$ channels was significantly higher than that of control species at -20 and -25 mV (Figure 4B). Additionally, the proportion change in post-venom Na^+ decay slope of grasshopper mouse channels was significantly lower than for control species (Figure 5B). Collectively, these results suggest that, although grasshopper mouse $Na_v1.4$ currents decay more slowly under baseline conditions, they are less perturbed by α toxins in *C. sculpturatus* venom than the controls. However, the biological significance of this finding is unclear because the absolute values of post-venom decay slopes are similar in all three species.

C. sculpturatus venom causes pain-related behavior, muscle spasms and death in house mice, but has little effect on grasshopper mice (Rowe & Rowe 2008). *Ex vivo* analyses showed that *C. sculpturatus* venom initially increased the force of contraction, and then blocked contraction in house mouse muscle, while having virtually no effect on grasshopper mice. While the current study shows that rat $Na_v1.4$ channels are significantly more sensitive to the α- and β-toxin effects of *C. sculpturatus* venom than grasshopper mice, grasshopper mice are not completely resistant to these effects. Moreover, house mice are only slightly more sensitive to β toxins than grasshopper mice. These findings suggest two critical points. First, a reduction in the sensitivity of grasshopper mouse $Na_v1.4$ to α and β toxins will not likely provide their muscle with complete physiological resistance to scorpion venom. *Centruroides* venom targets a range of ion channels, including voltage-gated potassium, calcium, and chloride channels, as well as other receptors involved in neuromuscular function (Valdivia &

Possani 1998; Tytgat *et al.* 1999; Escalona & Possani 2013). Future work should focus on investigating other molecular targets of *C. sculpturatus* venom in grasshopper mice muscle. Second, although house mice are identical to rat $Na_v1.4$ channels at the highly conserved sites shown in Figure 1, there are other extracellular loops and less conserved regions in which house mice either carry unique amino acid substitutions or, at least, differ from rats and grasshopper mice. These house mouse $Na_v1.4$ structural variants may have arisen due to random mutations that accumulated in the CD-1 lineage during the creation and/or maintenance of pure genetic lines. Conversely, the variants may represent relicts of ancestral selective pressures from scorpions prevalent in areas where the wild ancestors of house mice evolved. Fossil records suggest that the earliest ancestors of the genus *Mus*, lived on the Indian subcontinent approximately 5.5 million years ago (Boursot *et al*. 1993, Suzuki *et al.* 2013), during a time when scorpions likely prevailed in the area.

Three distinct regions of the $Na_v1.4$ channel contribute to reduced venom sensitivity

My second goal was to identify the amino acids involved in reducing sensitivity to *C. sculpturatus* venom. Because grasshopper mice express several amino acid variants in their $Na_v1.4$ protein, I used models of molecular evolution to guide my search for functionally important residues. The branch-site model of nucleotide substitutions (CODEML package, PAML) predicted that seven amino acid residues might be under positive selection in the *Onychomys* lineage. Of these, I333 in DI S5-S6 and Y1182 in DIII S5-S6 are in extracellular loops close to known *Centruroides* toxin binding sites

were associated with a >65% probability of being under positive selection. While I333 is positioned in the α toxin receptor site, Y1182 lies close to known β toxin binding sites. It is noteworthy that the amino acids adjacent to I333 (E334) and Y1182 (K1183) are also unique to the *Onychomys* lineage. However, these residues were not predicted to be under positive selection by the relatively conservative parameters of the branch-site model.

To maximize the probability of identifying residues responsible for reducing toxin sensitivity, I used electrophysiology to test the role of two amino acids in DI (I333 and E334) two in DIII (Y1182 and K1183), and the C-terminus insert (unique to grasshopper mice) by creating a triple mutant construct. I expected to observe differences between mutant and wildtype channels in baseline properties because the amino acids exchanged between the constructs are located in conserved regions. Indeed, I found the triple mutant channels to activate at more hyperpolarized membrane potentials than wildtype grasshopper mouse channels under baseline conditions (Table 2, Figure 6A). These findings are significant because they indicate that substitutions in DI, DIII and the C-terminus make wildtype grasshopper mouse $Na_v1.4$ channels (and likely their myocytes) less excitable. In all other measured baseline properties, triple mutant $Na_v1.4$ channels were similar to grasshopper mouse channels.

In the presence of *C. sculpturatus* venom there was a small but significant effect on the voltage dependence of activation for both channels. In the triple mutant, there was a greater increase in conductance at negative voltage steps compared to wildtype grasshopper mouse channels (Figure 6B). However, the triple mutant and grasshopper mouse channels experienced similar levels of post venom decreases in peak Na^+

current amplitude at positive membrane potentials. Additionally, the triple mutant channels did not differ from grasshopper mouse channels in their sensitivity to α toxins i.e., the two channels had similar Na^+ current decay slopes and decay constants (tau). These findings indicate that the amino acid variants seen in DI S5-S6, DIII S5-S6 and the C-terminus play some role in alleviating the effects of β- but not α-toxins in the venom of *C. sculpturatus*. Future work should identify additional regions of the grasshopper mouse $Na_v1.4$ channel involved in reducing sensitivity to the β (effects on peak current) and α-toxin effects. Some of these undiscovered regions of importance may be shared between grasshopper mice and house mice; this would explain why house mouse $Na_v1.4$ channels are less sensitive to *C. sculpturatus* venom than rat. Whether shared mechanisms of resistance are a result of convergent evolutionary forces or genetic drift acting on in-bred lines of CD-1 house mice are merely a matter of conjecture until further research is conducted.

Effect of structural modifications on resistance to α and β toxins may be additive

Next, I investigated the individual contributions of DI, DIII and C-terminus modifications towards reducing the wildtype grasshopper mouse $Na_v1.4$'s sensitivity to β toxins. In the presence of venom, I found that the Di and Diii mutants were similar to the triple mutants (Figure 8B and 8C) in their voltage dependence of activation (β toxin effect), whereas the voltage dependence of activation for NoC channels was intermediate to grasshopper mice and triple mutants (Figure 8D). These results show that the amino acid variants in grasshopper mouse $Na_v1.4$ DI, DIII and C-terminal are all involved in reducing sensitivity to the effects of β toxins on voltage dependence of

activation. These findings are significant because they suggest that three separate, structurally modified regions in the grasshopper mouse $Na_v1.4$ channel may contribute to reduced sensitivity to *C. sculpturatus* venom.

Proposed mechanism of resistance: role of amino acids in DI, DIII and C-terminus

Amino acids in extracellular loops of Na_v channels can influence the dissociation constant (K_d) of β toxins. Cestèle *et al.* (1998) found that chimeric mutagenesis replacements of amino acids in the pore loop of DI and DIII dramatically increased toxin K_d. Therefore, I propose that the DI and DIII amino acid substitutions seen in the $Na_v1.4$ channels of grasshopper mice reduce sensitivity to β toxins by lowering toxin K_d. Further investigation of dissociation constants using isolated *C. sculpturatus* venom peptides will be necessary to empirically elucidate the exact mechanisms involved in reducing venom sensitivity.

Although previous studies have shown that the intracellular C-terminus of Na_v channels plays a crucial role in determining the rate of fast inactivation, there is no prior evidence that this region affects activation properties. My study is the first to show that amino acids in the C-terminus region of Na_v channels may influence the interaction of venom toxins with voltage sensors during activation. Computational protein models that can elucidate structure-function relationships would help to unravel potential mechanisms through which the C-terminus interacts with voltage sensors during activation and venom binding.

Physiological costs associated with modifications that reduce sensitivity to *C. sculpturatus* venom

Structural modifications to highly conserved proteins, even if beneficial, are often accompanied by physiological costs. One such example of an evolutionary cost is seen in garter snakes (*Thamnophis sirtalis*) that feed on toxic newts (*Taricha granulosa*). Garter snakes carry unique amino acid modifications in their $Na_v1.4$ proteins that make them resistant to tetrodotoxin (TTX), a Na^+ pore-blocking neurotoxin found in newts (Geffeney 2002). However, the amino acids substitutions that make garter snake $Na_v1.4$ channels resistant to TTX have adverse effects on both the conductance of $Na_v1.4$ channels and their kinetic properties (Yoshida 1994; Brodie & Brodie 1999). In my own system, I found baseline differences in the voltage dependence of activation of grasshopper mouse, house mouse and rat $Na_v1.4$ channels. Grasshopper mouse $Na_v1.4$ channels activated at significant more depolarized potentials than rat and house mouse channels. Further, I could empirically demonstrate that the same unique structural modifications in the grasshopper mouse $Na_v1.4$ that make these channels partially resistant to *C. sculpturatus* venom are also responsible (to varying degrees) for shifting the channel's baseline activation threshold.

I also found that grasshopper mouse and house mouse $Na_v1.4$ channels have slower baseline inactivation kinetics compared to rat channels. While this inhibition of inactivation in grasshopper mouse $Na_v1.4$ may represent a cost associated with reduced sensitivity to *C. sculpturatus* venom, my data shows that it does not arise from the unique structural modifications identified in this study. Therefore, I propose that improper association between grasshopper mouse and house mouse $Na_v1.4$ alpha

subunits with rat beta subunits affects their inactivation kinetics. However, if these differences in inactivation kinetics do represent a trade-off, it likely arises from amino acid variants shared between grasshopper mice and house mice that also have consequences for venom resistance.

Summary and Significance

My study is the first to sequence the complete coding region of the grasshopper mouse (*Onychomys* spp.) skeletal-muscle $Na_v1.4$ gene (*Scn4a*), and to report the effects of Arizona bark scorpion (*C. sculpturatus*) venom on heterologously expressed $Na_v1.4$ channels from southern grasshopper mice, house mice and rats. Using comparative sequence analyses, molecular biology and electrophysiological recordings, I show that *C. sculpturatus* venom induces α- and β-toxin effects on rat $Na_v1.4$, disrupting channel activation and inactivation. However, as predicted, grasshopper mouse $Na_v1.4$ is significantly less sensitive to certain effects of these toxins. Further, I show that the molecular mechanism of reduced sensitivity involves amino acid variants at three distinct locations within the channel protein: two major and one minor region of contribution. These findings are significant because there are no other examples of ion channels that are resistant to distinct classes of peptide toxins simultaneously targeting different gating mechanisms (activation, inactivation), nor are there examples of resistance that involve structural modifications to multiple regions within a channel protein. Further, I empirically demonstrate that structural modifications to $Na_v1.4$ proteins result in significant trade-off to their functional properties that may represent physiological costs to muscle excitability. More broadly, the discovery of specific amino

acids influencing the voltage range over which ion channels activate may be important in the search for therapeutic targets for treating neuromuscular disorders that affect myocyte excitability.

REFERENCES

REFERENCES

Al-Sabi A, McArthur J, Ostroumov V, French RJ. 2006. Marine toxins that target voltage-gated sodium channels. *Marine Drugs* 4(3):157–192.

Armstrong CM. 1981. Sodium channels and gating currents. *Physiological Reviews* 61(3):644–683.

Bosmans F & Tytgat J. 2007. Voltage-gated sodium channel modulation by scorpion alpha-toxins. *Toxicon* 49(2):142–158.

Bosmans F, Martin-Eauclaire MF & Swartz KJ. 2008. Deconstructing voltage sensor function and pharmacology in sodium channels. *Nature* 456(7219):202–208.

Boursot P, Auffray JC, Britton-Davidian J, Bonhomme F. 1993. The evolution of house mice. *Annual Review of Ecology and Systematics* 24(1993): 119–152.

Brodie, ED, III & Brodie ED, Jr. 1999. Costs of exploiting poisonous prey: evolutionary trade-offs in a predator-prey arms race. *Evolution* 52(2):626–631.

Cahalan MD. 1975. Modification of sodium channel gating in frog myelinated nerve fibres by *Centruroides sculpturatus* scorpion venom. *The Journal of Physiology* 244(2):511–534.

Campos FV, Chanda B, Beirão PSL & Bezanilla F. 2008. Alpha-scorpion toxin impairs a conformational change that leads to fast inactivation of muscle sodium channels. *The Journal of General Physiology* 132(2):251–263.

Campos FV, Coronas FIV & Beirão PSL. 2004. Voltage-dependent displacement of the scorpion toxin Ts3 from sodium channels and its implication on the control of inactivation. *British Journal of Pharmacology* 142(7):1115–1122.

Catterall WA. 1979. Binding of scorpion toxin to receptor sites associated with sodium channels in frog muscle. Correlation of voltage-dependent binding with activation. *The Journal of General Physiology* 74(3):375–391.

Catterall WA. 2000. From ionic currents to molecular mechanisms: the structure and function of voltage-gated sodium channels. *Neuron* 26(1):13–25.

Catterall WA. 2010. Ion channel voltage sensors: structure, function, and pathophysiology. *Neuron* 67(6):915–928.

Catterall WA. 1980. Neurotoxins that act on voltage-sensitive sodium channels in excitable membranes. *Annual Review of Pharmacology* 20(1):15–43.

Catterall WA. 1986. Voltage-dependent gating of sodium channels: correlating structure and function. *Trends in Neurosciences* 9:7–10.

Catterall WA, Cestèle S, Yarov-Yarovoy V, Frank HY, Konoki K, *et al*. 2007. Voltage-gated ion channels and gating modifier toxins. *Toxicon* 49(2):124–141.

Cestèle S & Catterall WA. 2000. Molecular mechanisms of neurotoxin action on voltage-gated sodium channels. *Biochimie* 82(9-10):883–892.

Cestèle S, Qu Y, Rogers JC, Rochat H, Scheuer T, *et al*. 1998. Voltage sensor-trapping: enhanced activation of sodium channels by beta-scorpion toxin bound to the S3-S4 loop in domain II. *Neuron* 21(4):919–931.

Chanda B & Bezanilla F. 2002. Tracking voltage-dependent conformational changes in skeletal muscle sodium channel during activation. *The Journal of General Physiology* 120(5):629–645.

Clement H, Odell G, Zamudio FZ, Redaelli E, Wanke E, *et al*. 2007. Isolation and characterization of a novel toxin from the venom of the spider *Grammostola rosea* that blocks sodium channels. *Toxicon* 50(1):65–74.

Couraud F, Jover E, Dubois JM & Rochat H. 1982. Two types of scorpion receptor sites, one related to the activation, the other to the inactivation of the action potential sodium channel. *Toxicon* 20(1):9–16.

Denac H, Mevissen M & Scholtysik G. 2000. Structure, function and pharmacology of voltage-gated sodium channels. *Naunyn-Schmiedeberg's Archives of Pharmacology* 362(6):453–479.

Drukewitz SH, Fuhrmann N, Undheim EA, Blanke A, Giribaldi J, *et al*. 2018. A Dipteran's novel sucker punch: evolution of arthropod atypical venom with a neurotoxic component in robber flies (Asilidae, Diptera). *Toxins* 10(1):29–23.

Escalona MP & Possani LD. 2013. Scorpion beta-toxins and voltage-gated sodium channels: interactions and effects. *Frontiers in Bioscience* 18:572-587.

Fabre PH, Hautier L, Dimitrov D & Douzery EJ. 2012. A glimpse on the pattern of rodent diversification: a phylogenetic approach. *BMC Evolutionary Biology* 12(1):88-107.

Feldman DH & Lossin C. 2014. Na_V channel bench series: plasmid preparation. *MethodsX* 1:6–11.

Geffeney S. 2002. Mechanisms of adaptation in a predator-prey arms race: TTX-resistant sodium channels. *Science* 297(5585):1336–1339.

Goldin AL. 2002. Evolution of voltage-gated Na^+ channels. *The Journal of Experimental Biology* 205(5):575–584.

Goldin AL. 2003. Mechanisms of sodium channel inactivation. *Current Opinion in Neurobiology* 13(3):284–290.

Goldin AL, Barchi RL, Caldwell JH, Hofmann F, Howe JR, *et al*. 2000. Nomenclature of voltage-gated sodium channels. *Neuron* 28(2):365–368.

Gordon D, Savarin P, Gurevitz M & Zinn-Justin S. 1998. Functional anatomy of scorpion toxins affecting sodium channels. *Journal of Toxicology: Toxin Reviews* 17(2):131-159.

Guy HR & Seetharamulu P. 1986. Molecular model of the action potential sodium channel. *Proceedings of the National Academy of Sciences of the United States of America* 83(2):508–512.

Gwee MC, Nirthanan S, Khoo HE, Gopalakrishnakone P, Kini RM, *et al*. 2002. Autonomic effects of some scorpion venoms and toxins. *Clinical and Experimental Pharmacology & Physiology* 29(9):795–801.

Han H, Baumann K, Casewell NR, Ali SA, Dobson J, *et al*. 2017. The cardiovascular and neurotoxic effects of the venoms of six bony and cartilaginous fish species. *Toxins* 9(12):67–10.

Hanifin CT, Yotsu-Yamashita M, Yasumoto T & Brodie ED. 1999. Toxicity of dangerous prey: variation of tetrodotoxin levels within and among populations of the newt *Taricha granulosa*. *Journal of Chemical Ecology* 25(9):2161-2175

Hodgkin AL & Huxley AF. 1952. A quantitative description of membrane current and its application to conduction and excitation in nerve. *The Journal of Physiology* 117(4):500–544.

Horn R, Ding S & Gruber HJ. 2000. Immobilizing the moving parts of voltage-gated ion channels. *The Journal of General Physiology* 116(3):461–476.

Patterson RA. 1960. Physiological action of scorpion venom. *The American Journal of Tropical Medicine and Hygiene* 9(4):410-414.

Ismail M. 1995. The scorpion envenoming syndrome. *Toxicon* 33(7):825–858.

Isom L. 2014. Sodium Channel β1 Subunits: overachievers of the ion channel family. *Biophysical Journal* 106(2):1-11.

Kharrat R, Darbon H, Rochat H & Granier C. 1989. Structure/activity relationships of scorpion alpha-toxins. Multiple residues contribute to the interaction with receptors. *European Journal of Biochemistry/FEBS* 181(2):381–390.

Kirsch GE, Skattebøl A, Possani LD & Brown AM. 1989. Modification of Na channel gating by an alpha scorpion toxin from *Tityus serrulatus*. *The Journal of General Physiology* 93(1):67–83.

Klint JK, Senff S, Rupasinghe DB, Er SY, Herzig V, *et al*. 2012. Spider-venom peptides that target voltage-gated sodium channels: pharmacological tools and potential therapeutic leads. *Toxicon* 60(4):478–491.

Kruger LC & Isom LL. 2016. Voltage-gated Na^+ channels: not Just for conduction. *Cold Spring Harbor Perspectives in Biology* 8(6):a029264–15.

LoVecchio F & McBride C. 2003. Scorpion envenomations in young children in central Arizona. *Journal of Toxicology: Clinical Toxicology* 41(7):937–940.

Mantegazza M & Cestèle S. 2005. β-Scorpion toxin effects suggest electrostatic interactions in domain II of voltage-dependent sodium channels. *The Journal of Physiology* 568(1):13–30.

Marban E, Yamagishi T & Tomaselli GF. 1998. Structure and function of voltage-gated sodium channels. *The Journal of Physiology* 508(3):647–657.

Mebs D. 2001. Toxicity in animals. Trends in evolution? *Toxicon* 39(1):87–96.

Namadurai S, Yereddi NR, Cusdin FS, Huang CLH, Chirgadze DY, *et al*. 2015. A new look at sodium channel β subunits. *Open Biology* 5(1):140192.

Nyakatura K & Bininda-Emonds OR. 2012. Updating the evolutionary history of Carnivora (Mammalia): a new species-level supertree complete with divergence time estimates. *BMC Biology* 10(1):12.

Possani LD, Merino E, Corona M, Bolivar F & Becerril B. 2000. Peptides and genes coding for scorpion toxins that affect ion-channels. *Biochimie* 82(9-10):861–868.

Possani LD, Becerril B, Delepierre M & Tytgat J. 1999. Scorpion toxins specific for Na^+ channels. *European Journal of Biochemistry/FEBS* 264(2):287–300.

Prashanth JR, Dutertre S & Lewis RJ. 2017. Pharmacology of predatory and defensive venom peptides in cone snails. *Molecular BioSystems* 13:2453–2465.

Prentis PJ, Pavasovic A & Norton RS. 2018. Sea anemones: quiet achievers in the field of peptide toxins. *Toxins* 10(1):36–15.

Rimsza ME, Zimmerman DR & Bergeson PS. 1980. Scorpion envenomation. *Pediatrics* 66(2):298–302.

Rogers JC, Qu Y, Tanada TN, Scheuer T & Catterall WA. 1996. Molecular determinants of high affinity binding of α-scorpion toxin and sea anemone toxin in the S3-S4 extracellular loop in domain IV of the Na^+ channel alpha subunit. *The Journal of Biological Chemistry* 271(27):15950–15962.

Rowe AH & Rowe MP. 2008. Physiological resistance of grasshopper mice (*Onychomys* spp.) to Arizona bark scorpion (*Centruroides exilicauda*) venom. *Toxicon* 52(5):597–605.

Rowe AH & Rowe MP. 2006. Risk assessment by grasshopper mice (Onychomys spp.) feeding on neurotoxic prey (*Centruroides* spp.). *Animal Behaviour* 71(3):725–734.

Rowe, AH, Xiao, Y, Rowe, MP, Cummins, TR, & Zakon, HH, 2013. Voltage-gated sodium channel in grasshopper mice defends against bark scorpion toxin. *Science*, 342(6157):441–446.

Rowe, AH, Xiao, Y, Scales, J., Linse, KD, Rowe, MP, Cummins, TR, & Zakon, HH. 2011. Isolation and characterization of CvIV4: A pain inducing α-scorpion toxin. PloS one, 6(8): e23520.

Indumathi SM & Khora SS. 2017. Sub-cellular localization of tetrodotoxin in the tissues of the pufferfish *Takifugu oblongus*. *Indian Journal of Pharmaceutical Education and Research* 51(3):407–411.

Saporito RA, Donnelly MA, Spande TF & Garraffo HM. 2011. A review of chemical ecology in poison frogs. *Chemoecology* 22(3):159–168.

Sheumack DD, Howden ME, Spence I & Quinn RJ. 1978. Maculotoxin: a neurotoxin from the venom glands of the octopus *Hapalochlaena maculosa* identified as tetrodotoxin. *Science* 199(4325):188–189.

Silva JR & Goldstein SAN. 2013. Voltage-sensor movements describe slow inactivation of voltage-gated sodium channels I: wild-type skeletal muscle $Na_V1.4$. *The Journal of General Physiology* 141(3):309–321.

Smith RD & Goldin AL. 1998. Functional analysis of the rat I sodium channel in *Xenopus* oocytes. *The Journal of Neuroscience* 18(3):811–820.

Springer MS, Meredith RW, Gatesy J, Emerling CA, Park J, *et al.* 2012. Macroevolutionary dynamics and historical biogeography of primate diversification inferred from a species supermatrix. *PLoS ONE* 7(11):e49521.

Stühmer W, Conti F, Suzuki H, Wang X, Noda M, *et al.* 1989. Structural parts involved in activation and inactivation of the sodium channel. *Nature* 339(6226):597–603.

Suzuki H, Nunome M, Kinoshita G, Aplin KP, Vogel P, Kryukov AP, Jin ML, Han SH, Maryanto I, Tsuchiya K & Ikeda H. 2013. Evolutionary and dispersal history of Eurasian house mice *Mus musculus* clarified by more extensive geographic sampling of mitochondrial DNA. *Heredity* 111(5): 375-390.

Tejedor FJ & Catterall WA. 1988. Site of covalent attachment of alpha-scorpion toxin derivatives in domain I of the sodium channel alpha subunit. *Proceedings of the National Academy of Sciences of the United States of America* 85(22):8742–8746.

Thomsen WJ & Catterall WA. 1989. Localization of the receptor site for alpha-scorpion toxins by antibody mapping: implications for sodium channel topology. *Proceedings of the National Academy of Sciences of the United States of America* 86(24):10161–10165.

Tytgat J, Chandy KG, Garcia ML, Gutman GA, Martin-Eauclaire MF, *et al*. 1999. A unified nomenclature for short-chain peptides isolated from scorpion venoms: alpha-KTx molecular subfamilies. *Trends in Pharmacological Sciences* 20(11):444–447.

Valdez-Cruz NA, Dávila S, Licea A, Corona M, Zamudio FZ, *et al.* 2004. Biochemical, genetic and physiological characterization of venom components from two species of scorpions: *Centruroides exilicauda* Wood and *Centruroides sculpturatus* Ewing. *Biochimie* 86(6):387–396.

Valdivia HH & Possani LD. 1998. Peptide toxins as probes of ryanodine receptor structure and function. *Trends in Cardiovascular Medicine* 8(3):111–118.

van der Meijden A, Koch B, van der Valk T, Vargas-Muñoz LJ, Estrada-Gómez S. 2017. Target-specificity in scorpions; comparing lethality of scorpion venoms across arthropods and vertebrates. *Toxins* 9(10):312–10.

Vandendriessche T, Abdel-Mottaleb Y, Maertens C, Cuypers E, Sudau A, *et al.* 2008. Modulation of voltage-gated Na^+ and K^+ channels by pumiliotoxin 251D: a “joint venture” alkaloid from arthropods and amphibians. *Toxicon* 51(3):334–344.

Vandendriessche T, Olamendi-Portugal T, Zamudio FZ, Possani LD, Tytgat J. 2010. Isolation and characterization of two novel scorpion toxins: The α-toxin-like CeII8, specific for $Na_v1.7$ channels and the classical anti-mammalian CeII9, specific for $Na_v1.4$ channels. *Toxicon* 56(4):613-23.

Wang J, Yarov-Yarovoy V, Kahn R, Gordon D, Gurevitz M, *et al.* 2011. Mapping the receptor site for alpha-scorpion toxins on a Na^+ channel voltage sensor. *Proceedings of the National Academy of Sciences of the United States of America* 108(37):15426–15431.

Watt DD & Simard JM. 2008. Neurotoxic proteins in scorpion venom. *Journal of Toxicology: Toxin Reviews* 3(2-3):181–221.

Webber MM & Graham MR. 2013. An Arizona bark scorpion (*Centruroides sculpturatus*) found consuming a venomous prey item nearly twice its length. *Western North American Naturalist* 73(4):530–532.

Williams BL, Lovenburg V, Huffard CL & Caldwell RL. 2011. Chemical defense in pelagic octopus paralarvae: tetrodotoxin alone does not protect individual

paralarvae of the greater blue-ringed octopus (*Hapalochlaena lunulata*) from common reef predators. *Chemoecology* 21(3):131–141.

Yang S, Xiao Y, Kang D, Liu J, Li Y, *et al.* 2013. Discovery of a selective $Na_V1.7$ inhibitor from centipede venom with analgesic efficacy exceeding morphine in rodent pain models. *Proceedings of the National Academy of Sciences of the United States of America* 110(43):17534–17539.

Yoshida S. 1994. Tetrodotoxin-resistant sodium channels. *Cellular and Molecular Neurobiology* 14(3):227–244.

Zhang, J. 2005. Evaluation of an improved branch-site likelihood method for detecting positive selection at the molecular level. *Molecular Biology and Evolution*, *22*(12):2472–2479.

CHAPTER 2: Role of grasshopper mouse beta subunits in modulating the function of skeletal-muscle voltage-gated ion channels ($Na_v1.4$)

INTRODUCTION

Transmembrane proteins called voltage-gated sodium ion (Na_v) channels play a crucial role in mediating action potentials in the electrically excitable cells of nerves and muscles (Hodgkin & Huxley 1952). The mammalian Na_v protein complex is heterotrimeric; it comprises a large, heavily glycosylated, pore-forming alpha subunit (~ 260 kD) and two smaller accessory proteins called beta subunits (~36 kD) (Catterall 2000; Yu & Catterall 2003). The four homologous domains (DI-DIV) of a single alpha subunit polypeptide undergo complex three dimensional folding to form a transmembrane channel that is highly selective for Na^+ ions (Noda *et al.* 1984; Catterall 2000; Goldin 2002). While the expression of alpha subunit protein alone can produce functional Na^+ currents in heterologous cells (Goldin *et al.* 1986; Noda *et al*. 1986; Catterall 2000), to achieve physiologically relevant gating kinetics and current density, co-expression with beta subunits is essential (Winters & Isom 2016; Brackenbury *et al*. 2011)

Four beta subunit paralogs (*Scn1b- Scn4b*) in mammalian genomes encode the accessory beta 1 – beta 4 proteins (Isom *et al.* 1992; Isom *et al*. 1995; Morgan *et al.* 2000; Yu *et al.* 2003.). These beta subunit proteins, classified as type I transmembrane glycoproteins, share several structural features; they comprise of a large extracellular Ig-like N-terminus, one transmembrane segment and a short intracellular C-terminus region (Isom 2001; Winters & Isom 2016). Whereas beta 1 and beta 3 share 57%

sequence homology and associate non-covalently with the alpha subunit, beta 2 and beta 4 are 35% homologous, and associate with the alpha subunit through covalent disulfide bonds on the extracellular surface (Winters & Isom 2016; Chahine & O'Leary 2011). In heterologous cells, beta subunit co-expression increases cell surface expression of Na_v channels, changes the voltage range over which activation and inactivation occurs, as well as enhances fast inactivation and recovery from inactivation (McEwen *et al*. 2004; Isom *et al.* 1992; Isom *et al.* 1995; Patino *et al*. 2009; Yu & Catterall 2003). Indeed, the importance of beta subunits in regulating normal channel physiology can be seen in the range of human diseases that result from mutations in genes encoding beta subunits (Medeiros-Domingo *et al.* 2007; Dulsat *et al*. 2017; Patino *et al.* 2009; Escayg & Goldin 2010; Ogiwara *et al.* 2012).

Given their crucial role in regulating the excitability of cells, Na_v channels are often targets of offensive and defensive neurotoxic venoms produced across the animal kingdom. One such venom is produced by *Centruroides sculpturatus* or the Arizona bark scorpions in the family Buthidae (Bosmans & Tytgat 2007). When threatened, *C. sculpturatus* scorpions use their sharp stingers to deliver a cocktail of painful and lethal toxins into potential predators. Based on their effects on Na_v channels, these toxins are broadly classified as: 1) α toxins that impair the inactivation mechanism to prolong the channel's open state (Campos *et al.* 2008; Bosmans & Tytgat 2007; Thomsen & Catterall 1989; Kharrat *et al. 1*989), and 2) β toxins that promote premature activation of the channel (Mantegazza & Cestèle 2005; Cestèle & Catterall 2000; Escalona & Possani 2013). While α and β toxins bind to different regions of the $Na_v1.4$ alpha subunits, they work synergistically to cause prolonged depolarizations, and

spontaneous action potentials. In sensitive mammals (including human infants), the effects of venom on $Na_v1.4$ (expressed on skeletal muscles) can manifest as muscle paralysis, asphyxiation, respiratory failure and death.

Carnivorous rodents called grasshopper mice (*Onychomys torridus*) routinely encounter and consume bark scorpions in the wild (Rowe & Rowe 2006). In staged behavioral trials, grasshopper mice are not deterred by *C. sculpturatus* stings, having evolved physiological resistance to their venom (Rowe & Rowe 2006; Rowe & Rowe 2008). Previously, I sequenced the coding region of the gene that encodes the grasshopper mouse $Na_v1.4$ alpha subunit (*Scn4a*) and found several unique amino acid modifications in highly conserved regions of the channel. I demonstrated that these modifications made grasshopper mouse $Na_v1.4$ channels less sensitive to the α and β effects of *C. sculpturatus* venom compared to control channels representing venom sensitive rodents, *Rattus norvegicus* (rat) and *Mus musculus* (house mouse). However, grasshopper mouse $Na_v1.4$ channels were not completely resistant, exhibiting small, venom-induced shifts in their gating kinetics.

Given that grasshopper mice are completely resistant to natural *C. sculpturatus* stings and controlled venom injections (A. Rowe unpublished), what could explain their $Na_v1.4$'s partial sensitivity to toxins? One hypothesis (of many) is that grasshopper mouse $Na_v1.4$ alpha subunits require association with their own beta subunits to exhibit complete venom resistance. Because beta subunits are important contributors to Na_v function, association with species-specific beta subunits may be necessary to mimic physiologically relevant gating kinetics. Therefore, in this study, I tested the role of

grasshopper mouse beta subunits in modulating their $Na_v1.4$ activity and response to venom.

The expression of beta subunits is tissue specific and differentially regulated across development (Sutkowski & Catterall 1990). Two beta subunits are expressed in skeletal muscles: beta 1 and beta 4 (Makita *et al.* 1996, Yu *et al.* 2003). Studies show that beta 1 is highly expressed in skeletal muscles and its association has notable impacts on the voltage dependent properties of $Na_v1.4$ (Makita *et al.* 1996). Work on beta 4 association with $Na_v1.4$ is limited; however, the co-expression of beta 4 with $Na_v1.4$ channels in heterologous cells has significant effects on voltage dependence of activation (Yu *et al.* 2003.; Chahine & O'Leary 2011).

I detected structural modifications in the beta 1 and beta 4 by sequencing the genes that encode these proteins: *Scn1b* and *Scn4b*. However, my work shows that grasshopper mouse beta subunits affect neither the voltage dependent properties of the grasshopper mouse $Na_v1.4$ channels nor their response to venom.

MATERIALS AND METHODS

Molecular biology

I used the *Onychomys torridus* skeletal muscle tissue, RNA extraction and cDNA synthesis protocols described in Chapter 1. A gene specific reverse primer along with Oligo $d(T)_{20}$ primed the reverse transcription reaction for cDNA synthesis. I designed degenerate PCR primers by identifying conserved 5' and 3' regions in a *Scn1b/4b* alignment of multiple closely related species, and amplified the gene in one piece. Then I ran the amplified fragments on a 0.8% w/v agarose gel, excised and purified the

appropriate sized bands using Wizard SV Gel and PCR Clean-Up System (Promega). Such purified PCR samples from three *O. torridus* mice were sent to Genewiz for Sanger Sequencing of PCR products, sequence synthesis and cloning into pcDNA3.1+ expression vector. House mouse *Scn1b* and *Scn4b* were downloaded from the NCBI database and the full-length genes were synthesized and cloned into pcDNA3.1+ vector by Genewiz. Rat *Scn1b* and *Scn4b* channels are same as those described Chapter 1. I could not obtain rat *Scn4b* construct due to shortage of time. However, studies show that *Scn2b* shares structural and functional similarities with *Scn4b*, and likely modulates alpha subunit activity in similar ways (Winters & Isom 2016); therefore, I used the rat *Scn2b* gene in place of rat *Scn4b* for all experiments in this chapter. To synthesize Capped mRNA (cRNA) from both plasmid constructs, I used the mMESSAGE mMACHINE™ T7 Transcription Kit by ThermoFisher Scientific. Plasmids were linearized at the 3' end of the gene, past the stop codon, using NotI restriction enzyme. I used a nanodrop spectrophotometer (NanoDrop™ 2000, ThermoFisher Scientific) to quantify the synthesized cRNA.

Electrophysiology

Expressing *Scn1b* and *Scn4b* in *Xenopus laevis* oocytes: I purchased *Xenopus laevis* frog oocytes from Xenopus1, MI. I removed follicles surrounding oocytes using forceps and incubated these oocytes in ND-96 culture media as described in Chapter 1. Then I co-injected $Na_v1.4$ alpha subunit cRNA from grasshopper mice with cRNA generated from the grasshopper mouse *Scn1b* and *Scn4b* construct. To compare the effects of native beta subunits versus foreign beta subunits

on the grasshopper mouse $Na_v1.4$ activity, I compared the electrophysiological properties of this construct combination to: 1) grasshopper mouse $Na_v1.4$ alpha co-injected with rat beta 1 and rat beta 2, and 2) grasshopper mouse $Na_v1.4$ alpha co-injected with house mouse beta 1 and beta 2. For all construct combinations, alpha subunit and the two beta subunits were co-injected in a 1:1:1 ratio. All injections were performed as described in Chapter 1. Injected oocytes were stored at 16^{o}C for a maximum of 4 days from injection.

Recording: I used the standard two-electrode voltage clamp technique at room temperature to record sodium currents expressed by *Xenopus* oocytes after 8–48 hours of expression. Electrodes, solutions, recording chambers, instruments, and leak subtraction protocol were identical to those described in Chapter 1. Similarly, lyophilized crude *C. sculpturatus* venom extraction, storage and dilution protocols were identical to those described in Chapter 1.

Data extraction and analysis: I measured voltage dependence of activation, voltage dependence of Na^+ current decay slope, Na^+ current decay constant, voltage dependence of steady-state inactivation and recovery from inactivation. All electrophysiology protocols, methods of data extraction and statistical analysis are similar to those described in Chapter 1.

RESULTS

Sequence of genes encoding grasshopper mouse beta 1 and beta 4 subunits from skeletal muscle

To determine the role of accessory beta subunits in resistance to scorpion venom, I addressed two questions: 1) Do the grasshopper mouse beta 1 and beta 4 subunits differ from the beta subunits of control species (rats and house mice) in their effects on the grasshopper mouse $Na_v1.4$ channels? 2) Do grasshopper mouse beta 1 and beta 4 subunits play any role in reducing the $Na_v1.4$ channel's sensitivity to *C. sculpturatus* venom? To answer these questions, I sequenced the complete coding region of genes encoding beta 1 (*Scn1b*) and beta 4 (*Scn4b*) subunits from the skeletal muscles of grasshopper mice. By comparing these sequences to homologs from closely related species, I found that both *Scn1b* and *Scn4b* genes of grasshopper mice coded for interesting amino acid variants. For example, in the transmembrane segment of the beta 1 protein, there were four amino acid differences between the rat and grasshopper mouse homologues (figures not shown). One amino acid change from a polar uncharged asparagine (N) to another polar uncharged serine (S) at location 131 was unique to grasshopper mice. The beta 4 protein of grasshopper mouse differed from its rat homolog in eight amino acids. However, the most remarkable feature of the grasshopper mouse beta 4 was the presence of a unique amino acid insert located in the distal part of the protein's C-terminus (Figure 9, bolded amino acids). This insert comprised two polar uncharged amino acids (asparagine, N, and threonine, T), a negatively charged glutamic acid (E), and three positively charged arginines (R).

Beta 4	C- terminus
O. torridus	SSSGNDNTENGLPGSKAEEKPPT**NETRRR**RH
Deer mouse	SSSGNDNTENGLPGSKAEEKPPT------KV
Chinese hamster	SSSGNDNTENGLPGSKAEEKPPT------KV
Prairie vole	SSSGNDNTENGLPGSKAEEKPPT------KV
House mouse	SSSGNDNTENGLPGSKAEEKPPT------KV
Rat	SSSGNDNTENGLPGSKAEEKPPT------KV
Human	SSSGNDNTENGLPGSKAEEKPPS------KV

Figure 9: A partial multi-species amino acid sequence alignment of the beta 4 protein. Bolded region shows the C-terminus insert unique to *O. torridus* grasshopper mice.

Electrophysiological properties of GaGb, GaMb and GaRb channels

To test the influence of species-specific beta subunits on the grasshopper mouse $Na_v1.4$'s alpha subunit activity and response to venom, I compared the grasshopper mouse $Na_v1.4$ alpha subunit co-expressed with grasshopper mouse beta 1 and beta 4 (referred to as GaGb) to grasshopper mouse $Na_v1.4$ alpha co-expressed with: 1) rat beta 1-beta 2 (GaRb) and 2) house mouse beta 1-beta 4 (GaMb) subunits. I co-expressed all alpha-beta combinations in *Xenopus laevis* oocytes and used the two-electrode voltage clamp technique to measure their electrophysiological properties before and after the application of whole *C. sculpturatus* venom.

Voltage dependence of activation

In the absence of venom, there was a significant difference in the V_{50} parameters of GaGb and GaMb channels (2.39 mV difference; $z = 5.11$; $P << 0.01$, Table 4) but not GaGb and GaRb channels (0.96 mV difference; $z = 1.58$; $P = 0.11$, Table 4). These

data suggest that GaMb channels have a lower baseline excitability than GaGb and GaRb channels. However, due to small sample sizes and overlapping 1*SEs in Figure 10A, these results must be interpreted with caution.

Effect of *C. sculpturatus* venom on voltage dependence of activation: β toxins in *C. sculpturatus* venom induce a hyperpolarized shift in the voltage dependence of $Na_v1.4$ activation (and V_{50}). Because the post-venom shift in the normalized conductance for all channels was negligible (Figure 10B-D), I fit the post-venom data to a single Boltzmann equation. As expected, venom did not significantly shift the V_{50} values of any of the channels (Table 4). This shows that β toxins in *C. sculpturatus* venom had a negligible effect on GaGb, GaRb and GaMb channels.

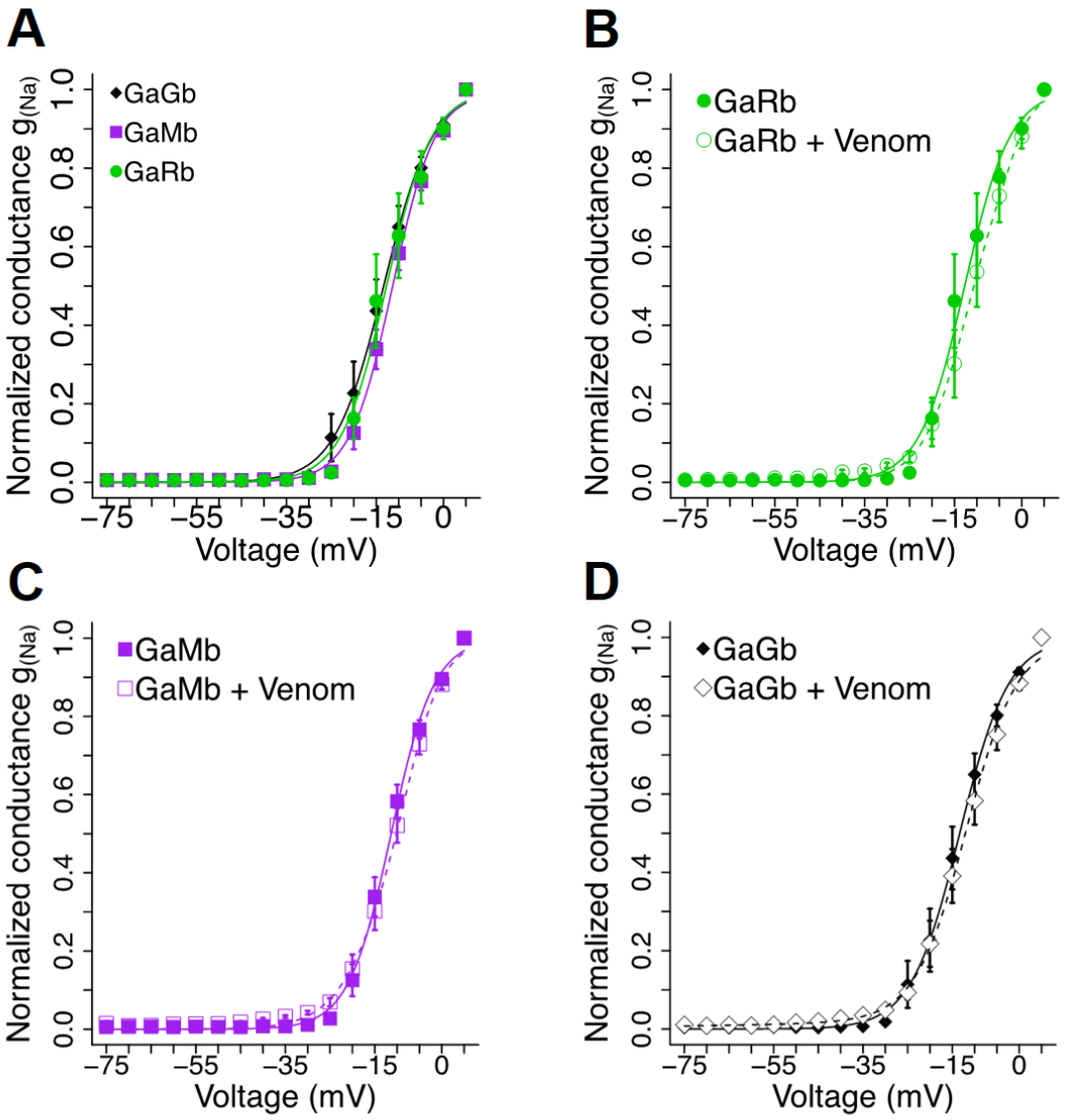

Figure 10: Influence of grasshopper mouse beta subunits on the voltage dependence of activation of grasshopper mouse $Na_v1.4$ channels. A) Normalized conductance-voltage relationship for GaGb (black) and GaMb (purple) and GaRb (green) before venom application. Effect of 0.15 µg/µl whole *C. sculpturatus* venom (open circles) on the normalized conductance voltage relationship of: B) GaRb (green) C) GaMb (purple) D) GaGb (black) channels. For all data collected with grasshopper mouse beta subunits, n = 10, for house mouse beta subunits n = 14 and for rat beta subunits n = 7.

Alpha+beta construct	V_{50}	K	n
GaGb	-13.34 ± 0.43	5.49 ± 0.37	10
GaGb + venom	-13.73 ± 0.39	5.86 ± 0.33	10
GaMb	-11.34 ± 0.24	5.49 ± 0.20	14
GaMb + venom	-11.34 ± 0.49	5.33 ± 0.42	14
GaRb	-12.70 ± 0.53	5.06 ± 0.00	7
GaRb + venom	-11.57 ± 0.64	5.88 ± 0.56	7

Table 4: Boltzmann parameters for normalized conductance vs. voltage relationships of GaGb, GaMb and GaRb channels. V_{50} is the voltage of half maximal activation, K is the slope of the relationship, P is the proportion of channels unaffected by venom and n is the number of oocytes sampled.

Effect of *C. sculpturatus* venom on peak current: β toxins in *C. sculpturatus* venom bind with Na_v channels and reduce peak Na^+ currents at depolarized voltage steps (see Chapter 1). This effect is demonstrated in the relationship between normalized peak current and voltage (Figure 11A and 11B). I found that GaGb, GaMb and GaRb channels experienced similar levels of peak current reduction in the presence of venom, especially at +5 mV (Figure 11C); a two-way repeated measures ANOVA found no significant differences between the peak Na^+ currents of GaGb and GaMb channels ($P = 0.41$; t-value = 0.41; df = 29) or GaGb and GaRb channels ($P = 0.65$; t-value = -0.74; df = 29) at voltages ranging from -5 mV to +45 mV in the presence of venom. These data show that GaGb, GaRb and GaMb channels do not differ in their sensitivities to β-*C. sculpturatus* toxins that reduce peak Na^+ current.

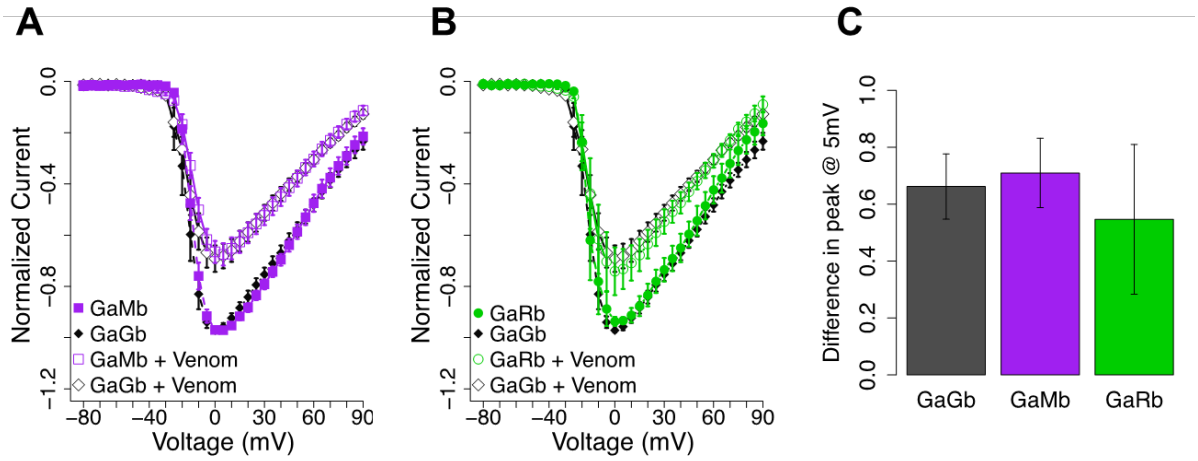

Figure 11: Effects of species- specific beta subunits on modulating $Na_v1.4$ activation and peak current in the presence of *C. sculpturatus* venom. All peak currents were normalized by pre-venom peak currents at different voltage steps. Voltage-current curves for: A) GaRb (green) and GaGb (black) B) GaMb (purple solid) and GaGb (black solid) before and after (open symbols) the application of venom. C) Difference between pre and post venom peak current observed at + 5 mV. All data represent mean ± 1SE.

Fast inactivation kinetics

α toxins in *C. sculpturatus* venom bind to the S3-S4 extracellular loop of DIV and disrupt the inactivation mechanism of Na_v channels. α-toxin bound channels experience prolonged ion flow well beyond the normal timeframe of inactivation (typically 4-5 ms after activation). To determine the α-toxin effect of *C. sculpturatus* venom on $Na_v1.4$, I applied a single depolarizing potential of 0 mV for 50 ms to oocytes expressing either GaGb, GaMb or GaRb channels, and then overlaid the current traces produced before and after the application of venom. In the absence of venom (solid lines), representative

current traces from all channels demonstrate rapid inactivation of Na^+ currents (Figure 12A). In contrast, the application of 0.15 µg/µl *C. sculpturatus* venom (dashed lines) slowed the decay of Na^+ current in GaGb, GaMb and GaRb channels, suggesting α-toxin inhibition of the fast inactivation mechanism in all three channels.

To quantify the effects of beta subunit association on fast inactivation kinetics, I measured Na^+ current decay constants (tau) and the slope of decay over a range of voltage steps (Figure 12B). A three-way repeated measured ANOVA suggested no baseline differences in the tau between GaGb and GaRb channels or GaGb and GaMb (F value = 0.293; df = 2, 29; P = 0.748). Similarly, there were no baseline differences in the decay slopes of GaGb and GaRb or GaMb channels (P > 0.1; df = 28, Figure 12C). These results suggest that species-specific beta subunits do not differentially affect $Na_v1.4$ alpha subunit's inactivation kinetics in the absence of venom.

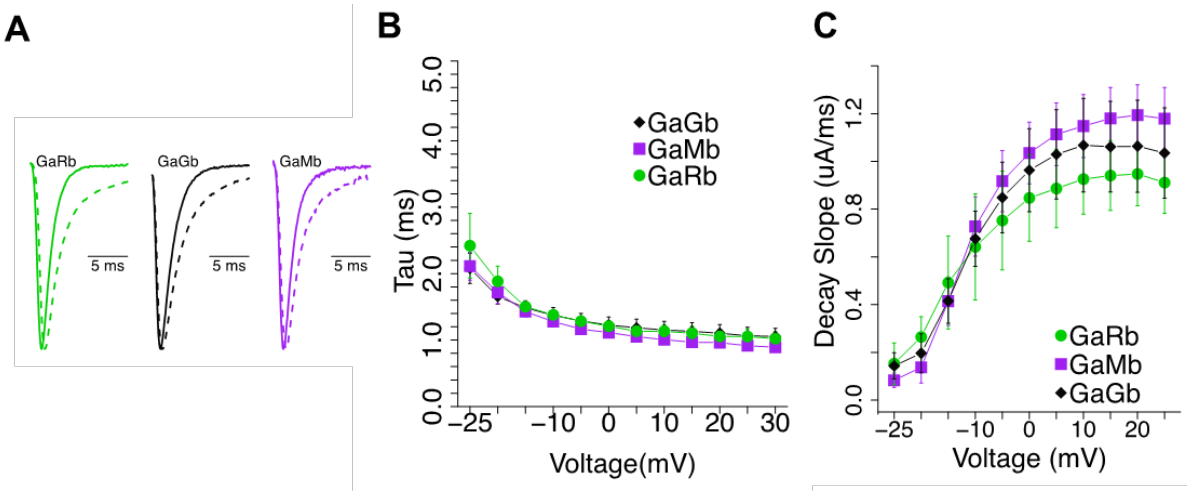

Figure 12: Effects of species-specific beta subunits on modulating $Na_v1.4$ alpha subunit baseline inactivation kinetics: A) Representative Na^+ current traces for GaGb (black), GaMb (purple) and GaRb (green) channels in the absence (solid line) and presence (dashed line) of venom. B) Tau plotted as a function of voltage for GaGb,

GaMb and GaRb channels. C) Na^+ current decay slope for GaGb, GaMb and GaRb channels.

Next, I applied *C. sculpturatus* venom to the oocytes expressing GaGb, GaMb and GaRb channels, and re-measured Na^+ current tau and decay slope. There were no differences in the tau value of GaGb, GaRb and GaMb channels in the presence of venom (Figure 13A-B; F-value = 1.5223; df = 2, 348; P = 0.2197). Venom reduced the slope of Na^+ current decay in all three channels (figure not shown), and the proportion change in slope before and after the application of venom was similar in the three channels (Figure 13C). A three-way repeated measures ANOVA showed that there were no significant differences in the proportion change in slope post venom for GaGb vs. GaRb (P = 0.29; df = 28; t-value = -1.078) or GaGb vs. GaMb (P = 0.38; df = 28; t-value = 0.89). Together, these results show that grasshopper mouse beta subunits do not influence the interaction of $Na_v1.4$ channels with *C. sculpturatus* venom in the *Xenopus* oocytes expression system.

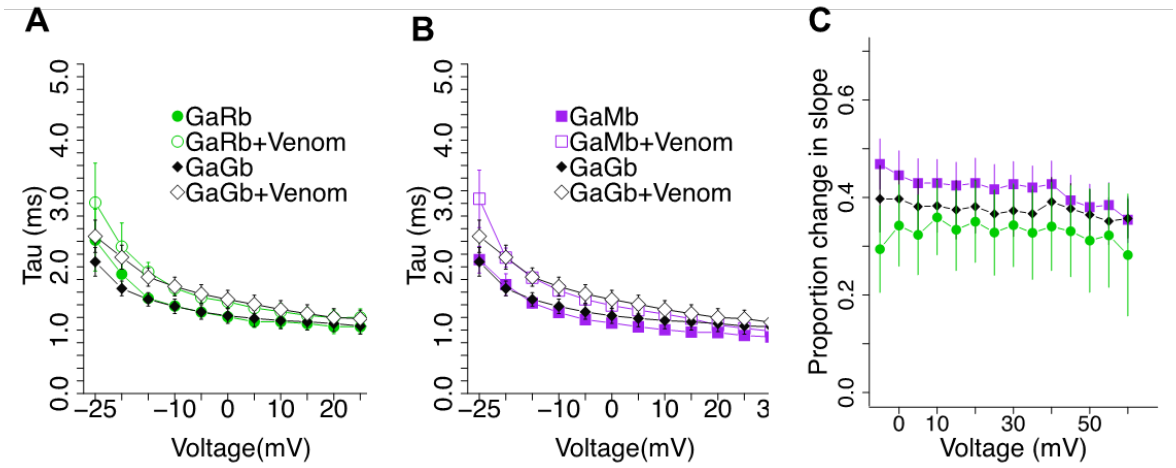

Figure 13: Effects of species-specific beta subunits on modulating $Na_v1.4$ alpha subunit's inactivation kinetics in the presence of *C. sculpturatus* venom. Na^+ current decay constant for A) GaGb (black) and GaRb channels (green). B) GaGb (black) and GaMb (purple) channels. C) Proportion change in post-venom Na^+ current decay slope calculated as described in Chapter 1. All points represent mean ± 1SE.

Voltage dependence of steady-state inactivation and recovery from fast inactivation

There were no differences between the steady-state inactivation or recovery from fast inactivation of GaGb, GaRb and GaMb either in the presence or absence of venom (overlapping ±1SE, figure not shown).

DISCUSSION

In Chapter 1, I showed that the grasshopper mouse $Na_v1.4$ alpha subunit has evolved amino acid modifications that reduced its sensitivity to *C. sculpturatus* venom. During all experiments in Chapter 1, I co-expressed $Na_v1.4$ alpha subunits with rat beta

subunits in order to make specific comparisons between alpha subunits of grasshopper mice and control species. Although it is well known that beta subunits can modulate the gating kinetics of alpha subunits, few studies have examined the consequences of co-expressing Na_v channels with their species-specific beta subunits in heterologous cells. In this chapter I examined the role of grasshopper mouse beta subunits in modulating the grasshopper mouse $Na_v1.4$ channel's baseline functional properties and response to *C. sculpturatus* venom.

In mammals, each Na_v alpha subunit protein associates with up to two beta subunits either through non-covalent interactions (with beta 1 and beta 3) or covalent di-sulfide bonds (with beta 2 or beta 4) (Chahine & O'Leary 2011; Winters & Isom 2016). These alpha-beta subunit associations are specific to different tissues and developmental stages. Skeletal muscles express only one Na_v alpha subunit protein ($Na_v1.4$) and at least two beta subunits: beta 1 and beta 4 (Goldin *et al.* 2000; Yu *et al.* 2003; Sutkowski & Catterall 1990; Chahine & O'Leary 2011). Although their *in vivo* associations with the $Na_v1.4$ alpha subunits are poorly understood, beta 1 and beta 4 proteins have proven effects on the gating properties and excitability of $Na_v1.4$ channels in heterologous cell lines (Makita *et al.* 1996; Yu *et al.* 2003.; Aman *et al.* 2009; Wallner *et al.* 1993). Using comparative sequence analyses, I found several amino acid differences between the beta 1 subunits of grasshopper mice versus rat and house mice. One amino acid change in the transmembrane segment of beta 1, N131 to S131, was unique to grasshopper mice. Both serine (S) and asparagine (N) are polar uncharged amino acids, but differ slightly in their side chains.

Unlike the beta 1 protein, the beta 4 protein of grasshopper mice did not carry any unique amino acid substitutions in its extracellular or transmembrane segments. However, I found a unique amino acid insert in the C-terminus region of the beta 4 protein. Of the six amino acids in this insert, there were three positively charged arginines (R), one negatively charged glutamic acid (E) and two polar uncharged residues (asparagine, N, and threonine, T). Presence of charged amino acids in the insert suggests that this region could interact electrostatically with the intracellular components of the $Na_v1.4$ alpha subunits, and/or modify the function of channel components that work from the intracellular end (e.g. the intracellular inactivation gate of Na_v channels). Therefore, I predicted that the structural modifications in both beta 1 and beta 4 proteins of grasshopper mice would influence their association with the alpha subunit and alter baseline functional properties of the channel. Additionally, there is evidence to suggest that beta 1 associates with Na_v channels on the extracellular surface, and in a region that likely overlaps with scorpion α-toxin binding sites on the alpha subunit (Makita 1996). Therefore, my second prediction was that structural modifications in grasshopper mice beta subunits would result in conformational changes that affect the interaction of Na_v channels with *C. sculpturatus* toxins.

Contrary to my predictions, I found that grasshopper mouse beta 1 and beta 4 subunits play no specific role in shaping the baseline or post-venom electrophysiological properties of grasshopper mouse $Na_v1.4$ channels in the *Xenopus* oocyte expression system. These findings suggest that minor changes to the side chain of certain amino acids (e.g. asparagine, N to serine, S) in transmembrane segments of beta 1 do not affect the alpha-beta associations in significant ways.

Similarly, the presence of a unique C-terminus insert in the grasshopper mouse beta 4 protein does not affect the baseline properties of $Na_v1.4$ channels or their response to venom. These findings were surprising because the insert is absent from the beta 4 sequence of closely related rodents (Figure 9), implying its association to a specific role in grasshopper mice. Additionally, the presence of charged amino acids in this region strongly indicates some physiological role. Future studies should test the role of the beta 4 C-terminus insert in regulating other cellular processes unrelated to gating, e.g., channel trafficking or subcellular localization. Alternatively, the C-terminus insert's ability to modulate channel gating properties may be contingent on the presence of mammalian regulatory proteins or transcription factors that are absent in frog oocytes. To test this hypothesis, future work should express Na_v channels and their beta subunits in a mammalian expression system (e.g. human embryonic kidney cells or Chinese hamster ovary cells).

Lastly, the C-terminus of beta 4 subunits is known to regulate neuronal excitability though its role in open channel block or OCB (Barbosa *et al.* 2015; Lewis & Raman 2013). Although OCB has never been described in muscle tissue, it is likely that the beta 4 C-terminus insert of grasshopper mice has been co-opted to serve as an OCB. As described in Chapter 1, Na_v channels inactivate within a few milliseconds of activation by employing an intracellular loop that acts as a hinged lid (Stühmer *et al.* 1989; West *et al.* 1992; Armstrong 2006). Once inactivated, Na_v channels require prolonged hyperpolarization in order to reset to "resting state" and activate again. While the inactivation mechanism helps prevent excessive action potential generation in most excitable cells, certain neurons (e.g. cerebral Purkinje cells) are required to produce

rapid trains of action potentials for normal functioning. Therefore, rapidly firing neurons by-pass the inactivation gate by employing the beta 4 subunit to form an OCB (Raman & Bean 1999; Barbosa *et al.* 2015). The OCB competitively binds to the docking site of the inactivation gate in a voltage-dependent manner and prevents further influx of Na^+ ions. However, unlike the inactivation gate, the OCB detaches from its docking site at more depolarized potentials (i.e. at potentials when the channel should be refractory under classical inactivation conditions). The release of the OCB is accompanied by a resurgence of transient Na^+ currents that can cause surrounding Na_v channels to open and elicit the next action potential (Barbosa *et al.* 2015; Schiavon *et al.* 2006; Patel *et al.* 2015). Therefore, my hypothesis is that the C-terminus insert in beta 4 of grasshopper mice enables this subunit to act as an OCB and regulate myocyte excitability when the channels are under attack by toxins that impair the inactivation mechanism. If this hypothesis is supported, it will uncover a potentially novel mechanism of α toxin resistance. However resurgent currents cannot be detected in the *Xenopus* oocytes; resurgent currents are fast and transient and do not last long enough to surpass the large capacitive currents generated in the *Xenopus* oocyte expression system. Future experiments aimed at understanding the role of the beta 4 C-terminus insert in *C. sculpturatus* toxins resistance should use a different heterologous expression system (e.g. Human Embryonic Kidney cells) which can detect resurgent currents.

REFERENCES

REFERENCES

Aman TK, Grieco-Calub TM, Chen C, Rusconi R, Slat EA, *et al.* 2009. Regulation of persistent Na current by interactions between beta subunits of voltage-gated Na channels. *The Journal of Neuroscience* 29(7):2027–2042.

Armstrong CM. 2006. Na channel inactivation from open and closed states. *Proceedings of the National Academy of Sciences of the United States of America* 103(47):17991–17996.

Barbosa C, Tan Z-Y, Wang R, Xie W, Strong JA, *et al.* 2015. $Na_v\beta4$ regulates fast resurgent sodium currents and excitability in sensory neurons. *Molecular Pain* 11:60.

Bosmans F & Tytgat J. 2007. Voltage-gated sodium channel modulation by scorpion alpha-toxins. *Toxicon* 49(2):142–158.

Campos FV, Chanda B, Beirão PSL & Bezanilla F. 2008. Alpha-scorpion toxin impairs a conformational change that leads to fast inactivation of muscle sodium channels. *The Journal of General Physiology* 132(2):251–263.

Catterall WA. 2000. From ionic currents to molecular mechanisms: the structure and function of voltage-gated sodium channels. *Neuron* 26(1):13–25.

Cestèle S & Catterall WA. 2000. Molecular mechanisms of neurotoxin action on voltage-gated sodium channels. *Biochimie* 82(9-10):883–892.

Chahine M & O'Leary ME. 2011. Regulatory role of voltage-gated Na channel β subunits in sensory neurons. *Frontiers in Pharmacology* 2:70.

Dulsat G, Palomeras S, Cortada E, Riuró H, Brugada R, *et al.* 2017. Trafficking and localisation to the plasma membrane of Na v1.5 promoted by the β2 subunit is defective due to a β2 mutation associated with Brugada syndrome. *Biology of the Cell* 109(7):273–291.

Pedraza EM & Possani LD. 2013. Scorpion beta-toxins and voltage-gated sodium channels: interactions and effects. *Frontiers in Bioscience* 18:572-587.

Escayg A & Goldin AL. 2010. Sodium channel SCN1A and epilepsy: Mutations and mechanisms. *Epilepsia* 51(9):1650–1658.

Goldin AL. 2002. Evolution of voltage-gated Na(+) channels. *The Journal of Experimental Biology*, 205(5):575–584.

Goldin AL, Snutch T, Lübbert H, Dowsett A, Marshall J, *et al.* 1986. Messenger RNA coding for only the alpha subunit of the rat brain Na channel is sufficient for

expression of functional channels in *Xenopus* oocytes. *Proceedings of the National Academy of Sciences of the United States of America* 83(19):7503–7507.

Goldin AL, Barchi RL, Caldwell JH, Hofmann F, Howe JR, *et al.* 2000. Nomenclature of voltage-gated sodium channels. *Neuron* 28(2):365–368.

Hodgkin AL & Huxley AF. 1952. A quantitative description of membrane current and its application to conduction and excitation in nerve. *The Journal of Physiology* 117(4):500–544.

Horner BE & Taylor JM. 1965. Food habits and gastric morphology of the grasshopper mouse. *Journal of Mammalogy* 45(4):513-535.

Isom, LL, De Jongh KS, Patten DE, Reber BF, Offord J, *et al.* 1992. Primary structure and functional expression of the beta 1 subunit of the rat brain sodium channel. *Science* 256(5058):839–842.

Isom LL, Ragsdale DS, De Jongh KS, WestenBroek RE, Reber BF, *et al.* 1995. Structure and function of the β2 subunit of brain sodium channels, a transmembrane glycoprotein with a CAM motif. *Cell* 83(3):433–442.

Isom LL. 2001. Sodium channel β subunits: anything but auxiliary. *Neuroscientist* 7(1):42-54.

Brackenbury WJ & Isom LL. 2011 Sodium Channel β1 Subunits: Overachievers of the Ion Channel Family. *Frontiers in Pharmacology* 2:53.

Kharrat R, Darbon H, Rochat H & Granier C. 1989. Structure/activity relationships of scorpion alpha-toxins. Multiple residues contribute to the interaction with receptors. *European Journal of Biochemistry/FEBS* 181(2):381–390.

Lewis AH & Raman IM. 2013. Interactions among DIV voltage-sensor movement, fast inactivation, and resurgent Na current induced by the Na Vβ4 open-channel blocking peptide. *The Journal of General Physiology* 142(3):191–206.

Makita N, Bennett PB & George AL. 1996. Molecular determinants of beta 1 subunit-induced gating modulation in voltage-dependent Na^+ channels. *The Journal of Neuroscience* 16(22):7117–7127.

Mantegazza M & Cestèle S. 2005. β-Scorpion toxin effects suggest electrostatic interactions in domain II of voltage-dependent sodium channels. *The Journal of Physiology* 568(1):13–30.

McEwen DP, Meadows LS, Chen C, Thyagarajan V & Isom LL. 2004. Sodium channel β1 subunit-mediated modulation of $Na_V1.2$ currents and cell surface density Is dependent on interactions with contactin and ankyrin. *The Journal of Biological Chemistry* 279(16):16044–16049.

Medeiros-Domingo A, Kaku T, Tester DJ, Iturralde-Torres P, Itty A, *et al.* 2007. SCN4B-encoded sodium channel beta4 subunit in congenital long-QT syndrome. *Circulation* 116(2):134–142.

Morgan, K, Stevens EB, Shah B, Cox PJ, Dixon AK, *et al.* 2000. beta 3: an additional auxiliary subunit of the voltage-sensitive sodium channel that modulates channel gating with distinct kinetics. *Proceedings of the National Academy of Sciences of the United States of America* 97(5):2308–2313.

Noda M, Shimizu S, Tanabe T, Takai T, Kayano T, *et al.* 1984. Primary structure of *Electrophorus electricus* sodium channel deduced from cDNA sequence. *Nature* 312(5990):121–127.

Noda M, Ikeda T, Kayano T, Suzuki H, Takeshima H, *et al.* 1986. Existence of distinct sodium channel messenger RNAs in rat brain. *Nature* 320(6058):188–192.

Ogiwara I, Nakayama T, Yamagata T, Ohtani H, Mazaki E, *et al.* 2012. A homozygous mutation of voltage-gated sodium channel βI gene SCN1B in a patient with Dravet syndrome. *Epilepsia* 53(12):e200–e203.

Patel RR, Barbosa C, Xiao Y, Cummins TR. 2015. Human $Na_V1.6$ channels generate larger resurgent currents than human $Na_V1.1$ channels, but the $Na_V\beta4$ peptide does not protect either isoform from use-dependent reduction *PLoS ONE* 10(7):e0133485.

Patino GA, Claes LR, Lopez-Santiago LF, Slaf EA, Dondeti RS, *et al.* 2009. A functional null mutation of SCN1B in a patient with Dravet syndrome. *The Journal of Neuroscience*, 29(34):10764–10778.

Raman IM & Bean BP. 1999. Properties of sodium currents and action potential firing in isolated cerebellar purkinje neurons. *Annals of the New York Academy of Sciences* 868:93–96.

Rowe AH & Rowe MP. 2008. Physiological resistance of grasshopper mice (*Onychomys* spp.) to Arizona bark scorpion (*Centruroides exilicauda*) venom. *Toxicon* 52(5):597–605.

Rowe AH & Rowe MP. 2006. Risk assessment by grasshopper mice (*Onychomys* spp.) feeding on neurotoxic prey (*Centruroides* spp.). *Animal Behaviour* 71(3):725–734.

Schiavon E, Sacco T, Cassulini RR, Gurrola G, Tempia F, *et al.* 2006. Resurgent Current and Voltage Sensor Trapping Enhanced Activation by a beta-Scorpion Toxin Solely in $Na_v1.6$ Channel: significance in mice Purkinje neurons. *The Journal of Biological Chemistry* 281(29):20326–20337.

Stühmer W., Conti F, Suzuki H, Wang X, Noda M, *et al.* 1989. Structural parts involved in activation and inactivation of the sodium channel. *Nature* 339(6226):597–603.

Sutkowski EM & Catterall WA. 1990. Beta 1 subunits of sodium channels. Studies with subunit-specific antibodies. *The Journal of Biological Chemistry* 265(21):12393–12399.

Thomsen WJ & Catterall WA. 1989. Localization of the receptor site for alpha-scorpion toxins by antibody mapping: implications for sodium channel topology. *Proceedings of the National Academy of Sciences of the United States of America* 86(24):10161–10165.

Wallner M, Weigl L, Meera P & Lotan I. 1993. Modulation of the skeletal muscle sodium channel α-subunit by the β1-subunit. *FEBS letters* 336(3):535-539.

West JW, Patton DE, Scheuer T, Wang Y, Goldin AL, *et al.* 1992. A cluster of hydrophobic amino acid residues required for fast Na(+) channel inactivation. *Proceedings of the National Academy of Sciences of the United States of America* 89(22):10910–10914.

Winters JJ & Isom LL. 2016. Developmental and Regulatory Functions of Na+ Channel non-pore-forming β subunits. *Current Topics in Membranes* 78:315-351.

Yu FH & Catterall WA. 2003. Overview of the voltage-gated sodium channel family. *Genome Biology* 4(3), p.207.

Yu FH, Westenbroek RE, Silos-Santiago I, McCormick KA, Lawson D, *et al.* 2003. Sodium channel beta4, a new disulfide-linked auxiliary subunit with similarity to beta2. *Journal of Neuroscience* 23(20):7577-7585.

CONCLUDING REMARKS

The aim of my dissertation research was to understand the molecular and physiological mechanisms through which the muscles of grasshopper mice (*Onychomys torridus*) are resistant to lethal neurotoxins in *Centruroides sculpturatus* venom. My study is the first to sequence the complete coding region of the genes encoding the $Na_v1.4$ alpha, beta 1 and beta 4 subunits from skeletal muscles of grasshopper mice. Using electrophysiology recordings, I show that grasshopper mouse $Na_v1.4$ channels are significantly less perturbed than the sensitive $Na_v1.4$ channels of house mice and rats to *C. sculpturatus* α and β toxin effects. Further, I could employ an interdisciplinary approach; by integrating techniques from molecular evolution, bioinformatics and molecular genetics, I identified specific amino acid modifications in three separate regions of the grasshopper mouse $Na_v1.4$ alpha subunit that confer reduced sensitivity to *C. sculpturatus* venom. My work is the first to reveal that amino acids in the $Na_v1.4$ channel's C-terminus can play a role in setting the channel's activation threshold.

Interestingly, although the amino acid modifications that confer reduced sensitivity are located in conserved regions of the channel, they have minimal effects on the channel's baseline functional properties. My findings suggest that highly conserved and functionally important Na_v channel proteins can be modified to encode alternative phenotypes without compromising overall function. Such proteins could serve as therapeutic targets for the design of precision medications to treat neuromuscular disorders.