β4 PEPTIDE MEDIATED VOLTAGE-GATED SODIUM CHANNEL RESURGENT CURRENTS OF HUMAN Nav1.5 SODIUM CHANNEL EXPRESSED IN HEK293 CELLS INCREASE AFTER EXPOSURE TO PYRETHROID INSECTICIDES PERMETHRIN AND CYPERMETHRIN

James O. Jackson, II

Submitted to the faculty of the University Graduate School
in partial fulfillment of the requirements
for the degree
Master of Science
in the Department of Pharmacology and Toxicology
Indiana University

August 2018

Accepted by the Graduate Faculty of Indiana University, in partial fulfillment of the requirements for the degree of Master of Science.

Master's Thesis Committee

Theodore R. Cummins, Ph.D., Chair

Nikolay Brustovetsky, Ph.D.

Alexander Obukhov, Ph.D.

Patrick L. Sheets, Ph.D.

DEDICATION

This work is dedicated to my beautiful wife Adrienne, and my wonderful children Tripp and AJ whose patience, support and sacrifice through the duration of this Master's work has given me the courage to finish this endeavor.

ACKNOWLEDGEMENTS

This thesis would not have been possible without the help, guidance, patience, support and sacrifice of many people whom I would like to acknowledge.

First I want to give honor and gratitude to the higher power within me and the universe for giving me focus in my distracted times, motivation in my unmotivated times, strength in my weak times and the desire to complete what I started when I felt like giving up would be easier times.

I would like to thank my mentor, Dr. Theodore Cummins. Thanks for believing in me enough to give me a chance to do research in your lab. Thank you for your patience in helping me see this endeavor to fruition. It has been great working with you all of these years! If not for your gentle guidance, and your encouragement I would not have completed this project. Thanks for giving me the space and time to complete this project. I am proud to be listed among your graduate students. I wish you the best in years to come.

I would like to thank Dr. Brustovetsky, Dr. Sheets, and Dr. Obukhov for being on my research and thesis committee. Your guidance, patience and understanding made it possible for me accomplish this difficult task. Thanks for your feedback and your willingness to work with me and help me complete this thesis. I am deeply indebted to you.

I would especially like to thank Dr. Yucheng Xiao. Thanks for your patience and your thoroughness in training me how to patch and walking with me, every step of the way, to the completion of my project. Thanks for always being available when I needed help with my research. You are a great person and a great teacher and I hope you continue to share your skills and knowledge with up and coming scientists. Best wishes to you and your family.

I would like to acknowledge everyone in the Cummins Lab, past and present that played a part in my training. Dr. Reesha Patel, thank you for your encouragement and holding my hand

through my early days of patching. Your skills, wisdom and your gentle and gracious teaching/training style helped me become a pretty good electrophysiologist. Dr. Cindy Nunez-Barbosa, thank you for your insights and your willingness in helping me trouble shoot my experiments and the patch rig during the days when nothing was working. Your kindness and lightheartedness made my bad days bearable. Dr. Zifan Pei, thank you for your quiet and patient disposition when helping me gain proficiency with electrophysiology techniques and learning how to patch on different rig set ups. Also thank you for all the times you watched over my cells so they would not be over grown and die before I could split them. Claudia, Agnes and Emily, thank you for your companionship during our shared graduate school journey. Hang in there, you will be finished in no time and ready to move on to the next chapter of your lives. I wish you all the best.

I would like to thank the Pharm/Tox department, namely Joanna Plew. Jo you are my hero! Thanks for all of your hard work in getting me through all the red tape and processes with the University Graduate School Office. Thanks for staying on top of me to make sure I was accomplishing what I needed to accomplish to successfully complete this program. You did a lot of foot work to help me hit all the necessary milestones needed to complete my thesis and graduate and for that I am grateful. I would also like to thank Amy Lawson and Lisa King for your help earlier on in my program. Thanks for making sure I was on track to complete this degree. Thanks Amy for all the advice you gave me about course work and research credit, what to take and when to take it. Thanks for getting me on the right track to graduate. Thanks Dan Smith for your assistance with helping me work out the logistics to fund this degree.

I would like to thank my kids. Thanks Tripp and AJ for your patience and encouragement during this journey. You witnessed me work through the course work, the patching, the data analysis and the writing of this thesis while juggling all of the other responsibilities in my life and

there were a lot of responsibilities. Thanks for giving me space and time to complete this project. Thanks for your sacrifice! Now, I look forward to making up that time that was lost to this work!

Last but certainly not least, I would like to thank my wonderful wife, Adrienne. You have inspired me and supported me through this entire endeavor. Thank you for the extra push when I did not feel like it. Thank you for the encouraging words when I hit a wall and felt demoralized. Thanks for your patience and sacrifice as I trudged through the work to finish this thesis. I thank you from the bottom of my heart with Love.

James O. Jackson, II

β4 PEPTIDE MEDIATED VOLTAGE-GATED SODIUM CHANNEL RESURGENT CURRENTS OF HUMAN Nav1.5 SODIUM CHANNEL EXPRESSED IN HEK293 CELLS INCREASE AFTER EXPOSURE TO PYRETHROID INSECTICIDES PERMETHRIN AND CYPERMETHRIN

Voltage-gated sodium channels (VGSCs) are transmembrane proteins responsible for the initiation of action potentials in excitable tissues by selectively allowing sodium ions (Na^+) to flow through the cell membrane. VGSC resurgent currents occur when an open channel blocker from the β4 subunit interacts with the α subunit, transiently blocking the movement of Na^+ across the membrane. VGSC subtype Nav1.5 channels are expressed in cardiac tissue and irregularities in their activity can lead to pathophysiological conditions like arrhythmias that can lead to death. Pyrethroid insecticides have been used widely in agriculture, vector control and households around the world for decades and since this is the case, human exposure to these products has increased dramatically. It is important to understand the effects of these insecticides on humans, including how these insecticides affect the heart. This thesis highlights the effects of pyrethroids on β4 peptide mediated Nav1.5 VGSC resurgent currents. The aims of this thesis were to 1) determine Nav1.5 channel activity and if activity changes with exposure to the vehicle (DMSO) used to dilute pyrethroids; 2) investigate the β4 peptide's effect on these Nav1.5 currents and if resurgent currents are produced; 3) investigate Nav1.5 channel activity when exposed to pyrethroids; and 4) investigate β4 peptide mediated VGSC resurgent current activity after exposure to pyrethroids. Standard whole-cell electrophysiology was used to determine electrophysiological and pharmacological properties of WT Nav1.5 currents. Results from these experiments showed that 1) Nav1.5 channel activity follows established understanding of VGSC: when depolarized a rapid and transient inward current is produced followed by a rapid inactivation; 2) DMSO did not affect activation and inactivation pattern; 3) the β4 peptide produced resurgent currents in Nav1.5; 4) pyrethroids alter electrophysiological

properties of Nav1.5 by prolonging inactivation; and 5) β4 peptide mediated resurgent currents are larger after exposure to pyrethroids. Overall, this thesis answers important questions regarding effects of pyrethroids on the cardiac VGSC and has implications for effects on health and highlights the necessity to be mindful of how pyrethroids are used in the future.

Theodore R. Cummins, Ph. D., Chair

Table of Contents

Curriculum Vitae

List of Figures

List of Abbreviations

α subunit	Alpha subunit
AP	Action Potential
β subunit	Beta subunit
β4	Beta four
CNS	Central Nervous System
CS	Chreoathatosis and Seziures – Intoxication Syndrome in rodents
DI – DIV	Domains of the Voltage-gated Sodium Channel
DMEM	Dulbecco's Modified Eagle Medium
G418	Neomycin
HEK293	Human Embryonic Kidney Cells
h_∞	H Infinity protocol
K^+	Potassium ions
MΩ	Mega Ohm
Na^+	Sodium ions
Nav	Voltage-gated Sodium Channel
SEM	Standard Error of the Mean
S1 – S6	Segments within Domains of the Voltage-gated Sodium Channel
T	Tremor – Intoxication Syndrome in rodents
TTX	Tetrodotoxin
TTX – S	Tetrodotoxin Sensitive
TTX – R	Tetrodotoxin Resistant
VGSC	Voltage-gated Sodium Channel
PNS	Peripheral Nervous System
WT	Wild type

Chapter One: Introduction and Background

Brief History of VGSC

Alan Hodgkin and Andrew Huxley were physiologists that conducted a series of experiments on the squid giant axon and they discovered that the transmission of action potentials along the axon of the nerve was dependent on the changes in permeability of positive ions, sodium (Na) and potassium (K), across the plasma membrane (Hodgkin and Huxley, 1952a). They also found that the membrane potential of the squid giant axon was involved in regulating sodium conductance and the kinetics of sodium current and that this was all controlled by what is now called a voltage-gated mechanism (Hodgkin and Huxley, 1952b). Years later, a selective Na current blocker, tetrodotoxin (TTX), from the liver of a puffer fish, was discovered to block Na currents in lobster giant axons (Narahashi et al., 1964). Similar findings were discovered when saxitoxin (STX) was added to a frog vagus nerve prep. Like TTX, STX, also a Na current blocker, selectively interacted with receptors on the frog vagus nerve prep to eliminate Na current (Narahashi et al. 1967; Hille, 1968). From these studies it was determined that Na current was voltage-dependent and conducted through individual channels. Tools like patch-clamp recording was invented and implemented to examine Na currents in small isolated neurons (Hamill et al., 1981; Sakmann et al., 1984) and has revolutionized the discovery and understanding of the different Na channel subtypes found in the human body. Although to date there are 9 sodium channel subtypes, this thesis will take a more in depth look at the Nav 1.5 isoform. But before we take a closer look at the Nav1.5, it is fitting to introduce the structure and function of the voltage-gated sodium channel in general.

Structure and Function of VGSC

Voltage-gated sodium channels are transmembrane proteins on many different types of tissues that participate in the influx of sodium ions and directly play a crucial role in propagating

action potentials in excitable tissues (Goldin, 2001). There are nine distinct alpha subunit genes that have been identified in mammals that encode VGSC isoforms (Nav.1.1 – Nav.1.9) which are found in different tissues (Goldin, 2002). For instance, Nav1.4 is largely expressed in skeletal muscle (Trimmer et al., 1989). Nav1.5 is mostly expressed in cardiac tissue (Rogart et al., 1989). Nav1.3, on the other hand, is predominantly expressed in immature neurons and downregulated in mature neurons (Beckh et al., 1989). Nav1.7, Nav1.8 and Nav1.9 are predominantly expressed in the peripheral nervous system (PNS). In contrast, in the mature central nervous system (CNS) neurons the predominant isoforms are Nav1.1, Nav1.2 and Nav1.6, however Nav1.1 and Nav1.6 are also expressed in the PNS (Trimmer et al., 2004).
The alpha subunit is the primary functional unit of the VGSC and is a 220-260 kDa polypeptide that can associate with auxiliary proteins including the four β subunits (Navβ1 – Navβ4) which are 30-40 kDa polypeptides. VGSC alpha subunit consists of four transmembrane domains (DI-DIV) linked by intracellular loops wherein each domain consists of six transmembrane segments (S1 – S6; see Figure 1.) (Noda et al., 1984). Each of the S4 segments are made up of four to eight positively charged amino acids (arginine and lysine) that act as voltage sensors and these S4 segments move within the membrane, in response to membrane voltage (or potential) changes, thus, creating various gating configurations that allows sodium ions to selectively flow inward through the channel (Noda et al., 1984; Catteral et al. 2000).

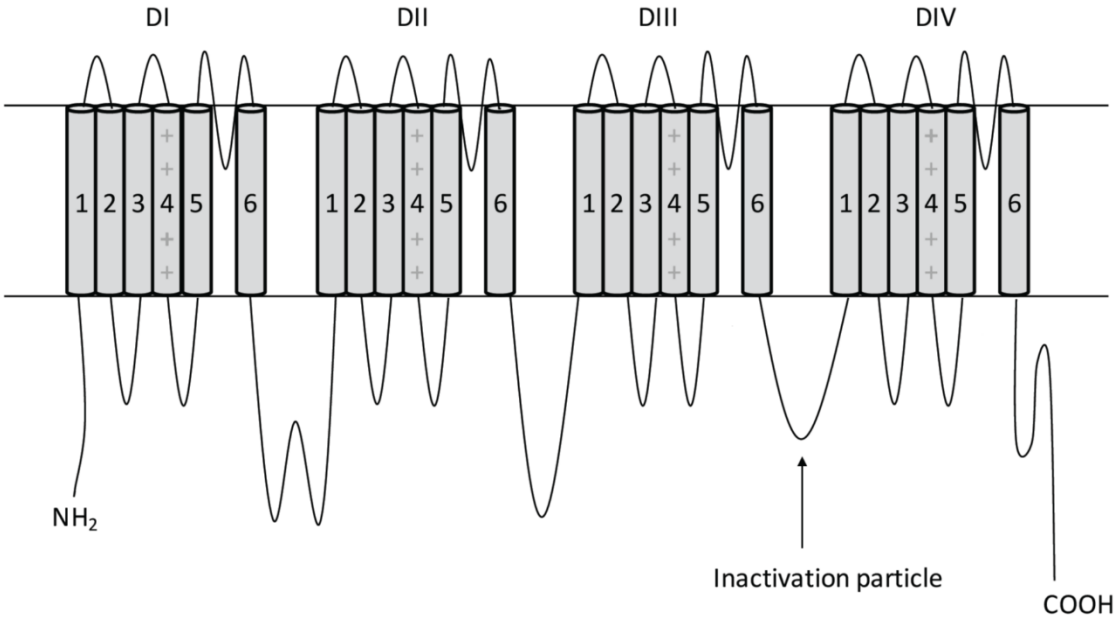

Figure 1. Linear schematic representation of the α subunit for the Nav1.5 voltage-gated sodium channel. The α subunit consists of four homologous transmembrane domains (DI—DIV). Each domain contains six transmembrane segments (S1—S6). S1-S4 serve as the voltage sensor of the channel and S5-S6 form the pore. This figure was adapted from Zifan Pei.

Simply put, VGSCs change between gating configurations that either allow sodium ions to flow through (open state) or do not allow sodium ions to flow through (inactive state and closed state) See figure 2. Closed refers to the configuration of the VGSCs at negative (or hyperpolarized) membrane potentials where the channel is available to open in the event the membrane changes to more positive (or depolarized) membrane potentials. In response to the depolarization of the membrane the VGSCs change to an open configuration and allow the selective inward movement of Na ions through the channel. Within roughly two milliseconds of opening the sodium currents are then terminated by channel inactivation. The typical mode of inactivation is fast inactivation where the cytoplasmic linker between DIII-DIV (see Figure 1), which includes the IFM (Isoleucine-Phenylalanine-Methionine) particle, also known as the inactivation particle, binds and occludes the pore which stops the inward flow of sodium ions (West et al. 992; Moorman et al., 1990). In one experiment a triple mutation of IFM to QQQ (glutamine) in Nav1.2, a brain VGSC was shown to slow down inactivation (West et al., 1992). Along similar lines another experiment a single point mutation of F (phenylalanine) to Q in hNav1.5 also slowed down the rate of inactivation (Bennett et al., 1995). The findings of these two experiments support the hypothesis that cytoplasmic linker between DIII and DIV and the IFM particle play an integral role in the inactivation of VGSCs.

However, fast inactivation is not the only type of inactivation. Studies show that there is also a slower component of inactivation, particularly in cardiac channels (Vilin et al., 1999). Slow inactivation happens when the membrane is depolarized for a long period of time and usually takes seconds to minutes for the channel to recover. Typically, once the channel is inactivated, by either mechanism, it becomes refractory and it requires repolarization of the membrane to recover before they are available again (Armstrong et al., 1974).

Nav1.5 Sodium Channels

The Nav1.5 VGSC is the predominant VGSC found in cardiac muscle (Rogart et al., 1989) and it plays a critical role in the initiation and propagation of action potentials throughout the heart. Similar to the other types of VGSCs, Nav1.5 undergoes conformational changes between ion-conducting (open) and non-conducting (inactive and closed) in response to change in membrane potential.

Cardiotoxicity has been reported as a result of pyrethroid intoxication. There is a specific cardiac sodium channel isoform (Nav 1.5) which has important clinical significance in that its mutation are implicated in malignant cardiac arrhythmias (Spencer et al., 2001). We predict that resurgent currents caused by Cypermethrin and Permethrin intoxication also play a role in malignant cardiac arrhythmias that can lead to health complications or death.

Resurgent Currents

Resurgent sodium currents were first discovered in cerebellar Purkinje neurons (Raman, et.al., 1997). They are produced when the open-channel blocker unbinds the pore during intermediate repolarizing potentials following depolarization. After depolarizing potentials open sodium channels some fraction of the channels can go through a blocked state that is faster than and different from classic fast inactivation. As the channel repolarizes to more negative or intermediate potentials the blocker unbinds and allows the movement of sodium ions through the pore producing resurgent current, after which the channels inactivate or deactivate (Raman et al., 2001) (see Figure 2). Rapid firing is permitted here because resurgent currents allow channels to cycle between open, blocked and unblocked states which bypasses fast inactivation that is important for the refractory period following an action potential. The rate of fast inactivation is a significant determinant of resurgent sodium current generation because it is believed that the open-channel blocker competes with the intrinsic fast inactivation particle

when binding to the channel pore. Studies using toxins and disease mutations have demonstrated that slower rates of fast inactivation enhances resurgent current generation (Jarecki, et al., 2010).

The first identified endogenous open channel blocker implicated in resurgent current generation is Navβ4. There are a few studies that demonstrate the significance of Navβ4 for the generation of resurgent cure. One study shows that Navβ4 – null mice do not have the ability to generate resurgent sodium current in medium spiny neurons of the striatum (Miyazaki, et al., 2014). Another study showed that knockdown of Navβ4 in dorsal root ganglion (DRG) sensory neurons decreased resurgent sodium current but when Navβ4 is overexpressed resurgent currents are increased in the Nav1.6 isoform (Barbosa, et al., 2015). Yet, another study showed that the knockdown of Navβ4 using small interfering RNA in cerebellar granule neurons completely wipes out resurgent sodium current, but interestingly, the resurgent current can be restored with the addition of a Navβ4 peptide (Bant, et al., 2010). However, co-expression of the VGSC alpha subunit and Navβ4 in HEK293 cells is not sufficient to produce resurgent sodium currents which suggests that other modulatory proteins and cellular background factors are critical. Although this is the case, the Navβ4 peptide, made up of the membrane proximal C-terminal portion of the Navβ4, $Navβ4_{154-167}$ (KKLITFILKKTREK), acts as an open channel blocker and produces resurgent sodium current in HEK293 cells (Greico, et al., 2005).

A depolarizing drive that approaches threshold for firing additional action potentials is caused by the momentary reopening of channels after the unbinding of the open-channel blocker. Studies show that channels that undergo open-channel block recover faster and are available sooner after an action potential spike decreasing the refractory period after an action potential (Theile, et al., 2011; Tan, et al., 2014). In fact, sodium currents have been shown to flow during the refractory period between action potentials and these currents are predicted to

enhance neuronal excitability (Raman, et al., 1999). Therefore, preventing resurgent current generation by knockdown of Navβ4 decreases spontaneous firing and repetitive firing with long depolarization stimuli (Barbosa, et al., 2015). These findings have also been demonstrated in modeling studies (Khaliq, et al., 2003).

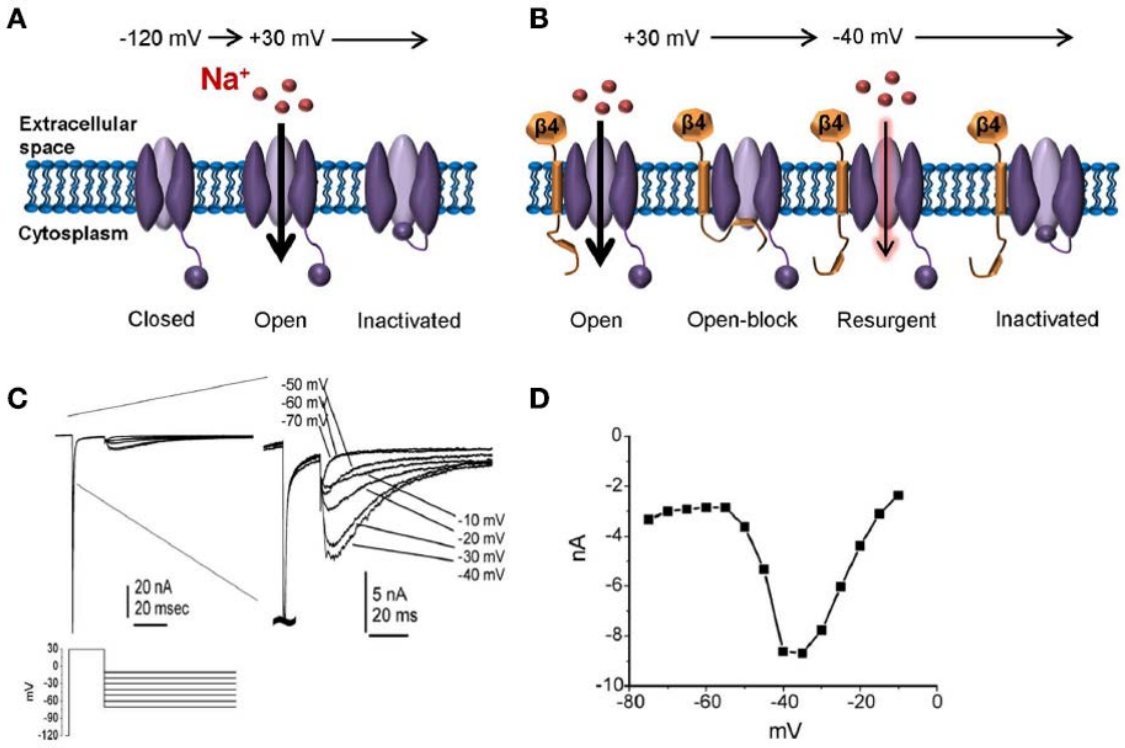

Figure 2. Resurgent sodium current. A, After a strong depolarization, sodium channels transition from the resting closed-state to open, allowing influx of sodium. Within milliseconds, the channel inactivates and remains inactivated until the membrane potential has been sufficiently hyperpolarized. This cycle of events underlies the action potential refractory period. B, Following a strong depolarization, a blocking particle (likely the C-terminal portion of the auxiliary Navβ4 subunit) can occlude the open-channel before the inactivation gate can bind, thus resulting in open-channel block. Following a hyperpolarization to an intermediate potential, the blocker is expelled resulting in an additional surge in sodium current. C, Representative resurgent sodium currents recorded from a large Nav1.8-null DRG neuron. The traces are magnified in the right panel to better see the resurgent currents. D, The voltage-dependence of the resurgent currents is shown by plotting the peak resurgent current amplitude against the repolarization pulse potential. Adapted from Theile and Cummins 2011).

Pyrethroid Insecticides

Pyrethrum is a substance extracted from the flowers of *Chrysanthemum cinerariaefolium* and *Chrysanthemum cineum* and has been recognized for its insecticidal properties since the 1800's when they were used by Caucasian tribes and in Persia to control body lice (Casida 1980). The six active insecticidal compounds of pyrethrum are called pyrethrins and are named pyrethrin I, pyrethrin II, cinerin I, cinerin II, jasmolin I and jasmolin II. These pyrethrins are localized in the secretory ducts of the seed bearing fruit, where they are protected from photodecomposition and isolated so they are not toxic to insects that come into contact with pyrethrum flowers (Casida 1980). The process to extract these pyrethins involves picking and drying the petals of the flowers and then grinding the petals into powder form and extracting the pyrethrins with hexane. After which, the hexane is evaporated and what is left is a dark substance containing 30% pyrethrins (Casida 1980). This substance can now be used as an insecticide with many different types of applications in different settings to knock down or control insect populations.

One of the disadvantages of the naturally occurring pyrethrum as an insecticide is that it is not stable in light and air, which limit its insecticidal effectiveness in crop protection and other insect control contexts where residual activity is paramount. The development of synthetic pyrethroids is the result of efforts to modify the structure of the natural pyrethrins in order to increase photo stability while retaining the potent and rapid insecticidal activity and relatively low acute toxicity of pyrethrum. The past several decades of research and development by the agrochemical industry, academic research laboratories and by the government have resulted in 1,000 different pyrethroid insecticides and a plethora of uses in agriculture, veterinary, medical and household pest control (Elliott 1989; Thatheyus et al. 2013). Synthetic pyrethroid development has involved structural modification and biological evaluation, so that the collection of present day commercial insecticides identified as pyrethroids are compounds that are several

steps removed from the pyrethrin structures. Most of these synthetic pyrethroids were discovered by sequentially replacing structural elements of the pyrethrins with novel structural moieties that were chosen to conserve the molecular shape and physical properties of the structure used as the template. Later, instead of using the pyrethrins as templates, subsequent stages used to develop new pyrethroids made use of the newly discovered synthetic pyrethroids with desirable insecticidal activity, stability and other properties as templates for the further design of new synthetic pyrethroids. For example the first wave of synthetic pyrethroids made use of pyrethrins as templates and one of the synthetic insecticides developed was permethrin, a Type I pyrethroid, after which, permethrin was used as a template to develop cypermethrin, a Type II pyrethroid.

Mode of Action of Pyrethroid Insecticides

The primary mode of action of pyrethroid in both insects and mammals is disruption of the VGSC function. Evidence points to the fact that pyrethroids bind to the α subunit of the VGSC. In a study that expressed Nav 1.2 α subunit in Chinese Hamster ovary cells (CHO cells) found that the presence of the α subunit was sufficient for pyrethroids to produce their characteristic effects on sodium channel function (Trainer et al., 1997). This conclusion is supported by another study that showed that pyrethroids altered the current produced by Nav 1.2 and Nav 1.8 α subunit expressed in oocytes in the absence of coexpression with β subunits (Smith et. al., 1998; Smith et. al., 2001). Interestingly, coexpression of the β1 subunit with Nav 1.2 increased the sensitivity of this channel compared with expression of Nav 1.2 alone indicating that the β subunit modulates the affinity of pyrethroid interaction with the channel (Smith et.al, 2001). Lastly, studies show that mutations in the VGSC α subunit of insects and mammals alters the sensitivity of VGSCs to effects of pyrethroids supporting the claim that pyrethroids interact with the α subunit (Lee et. al., 2001; Wang et. al., 2001).

Pyrethroids are known to alter the normal functioning of insect VGSC which are integral in mediating the transient increase in the sodium permeability of the nerve membrane that underlies the nerve action potential (Soderlund et. al., 1989). Pyrethroids slow the activation (or opening) of VGSCs and slow the rate of inactivation (or closing) of VGSCs. In addition they shift to more hyperpolarized potentials the membrane potential at which VGSCs initially activate (or open). The result is that the sodium channels open at more hyperpolarized potentials (i.e., after smaller depolarizing changes in membrane potentials) and remain open longer, allowing more sodium ions to cross and further depolarize the membrane. Studies show that in general, type II pyrethroids delay the inactivation of VGSCs significantly longer than the type I pyrethroids (Narahashi 1996). Type I pyrethroids keep channels open long enough to cause repetitive firing of action potentials while Type II pyrethroids keep channels open for so long that the membrane potential ultimately becomes depolarized so much that it can no longer generate action potentials, which is called depolarization dependent block (Soderlund et al, 2002). It is believed that these differences in prolongation of channel openings contribute to the differences between two distinct syndromes (see below) after exposure to Type I and Type II pyrethroids respectively (Ray 2001).

Pyrethroids act as contact poisons affecting the insect's nervous system by causing multiple action potentials in the nerve cells, primarily by delaying the closing of the VGSCs (Costa, 1997). Most insecticide products containing pyrethroids usually contain a synergist, such as piperonyl butoxide, which restricts an enzyme that insects use to detoxify the pyrethroid thereby making the insecticide more effective (Tomlin, 1994). Perturbation of sodium channel function by pyrethroids is stereospecific (Lund et. al., 1982). The sterioisomers that disrupt the VGSCs the most also have the most insecticidal activity (Ray, 2001). Pyrethroid exposure in

insects is predominately through the insect cuticle. Rapid absorption into the insect causes a disruption of neurotransmission causing paralysis and possibly death within seconds to minutes. Pyrethroid insecticides were introduced into common use for the control of insect pests in agricultural settings and in disease vectors over three decades ago. Their use had grown to represent 18% of the dollar value of the world insecticide market by 2002 (Pickett 2004). These Pyrethroid insecticides are not only effective in controlling agricultural pests, they are also used as a front line solution in efforts to combat malaria and other mosquito-borne diseases even with threats that there is already resistance forming in vector populations (Ranson et al. 2011). Pyrethroids are also used widely as ingredients in household insecticides and ectoparasite control products for domesticated pets. Pyrethroid use in agriculture and vector control has increased tremendously in recent years due to the reduced use of chlorinated, carbamate, and organophosphate pesticides causing an increased in human exposure to pyrethroids (Power et al., 2007). The unregulated use of these pesticides in the home environment increases the risk of exposure and potential adverse effects in the general population (Naeher et al. 2010).

Pyrethroid Intoxication in Mammals

Humans and other mammals quickly metabolize pyrethroid compounds to non-toxic substances so exposure to these compounds is usually unthreatening. However, reports of accidental exposure (occupational) and intentional poisoning (suicide attempts) due to pyrethroid insecticides are common (Chen et al., 1991).

Categorizing pyrethroid insecticides is complicated due to the existence of two distinct intoxication syndromes in mammals that are determined by different structural subgroups of the insecticide class. The first systematic description of the signs of pyrethroid intoxication in rats following either oral or intravenous dosing manifested the same syndrome which included hypersensitivity and aggression followed by tremors and convulsive twitching, coma and death

(Verchoyle et al. 1972). The observed difference between oral and intravenous dosing was the speed at which the symptoms of intoxication manifested. When the α-cyano-3-phenoxybenzyl moiety (α-cyano moiety) was added to the pyrethroid insecticide a different intoxication syndrome was identified. A landmark study reported salivation without lacrimation followed by jerking leg movements and progressive writhing convulsions, called choreoathetosis (Barnes et al. 1974).

A subsequent study described both intoxication syndromes following intravenous administration, thus establishing a taxonomy of pyrethroid intoxication in mammals that is still useful to the present (Verschoyle et al. 1980). The signs of intoxication first described for pyrethroids, hypersensitivity and aggression followed by tremors and convulsive twitching, coma and death (Verchoyle et al. 1972) was designated the T (tremor) syndrome. By contrast, the signs of intoxication produced by the pyrethroids with the α-cyano moiety, salivation without lacrimation followed by jerking leg movements and progressive writhing convulsions, called choreoathetosis (Barnes et al. 1974), was designated the CS (choreoathetosis with salivation) syndrome.

An alternative nomenclature (Type I and Type II) was proposed for groups of pyrethroids based on both their syndromes of intoxication, chemical structures, signs of poisoning in insects, and action on insect nerve preparations (Lawrence et al. 1982) (Gammon et al. 1981). Type II pyrethroid insecticides contain the α-cyano moiety (e.g. cypermethrin) whereas Type I pyrethroid insecticides are made up of compounds that are lacking the α-cyano moiety (e.g. permethrin) see figure 3. The Type I and Type II nomenclature is often used in a manner akin to the T and CS nomenclature, such that the Type I pyrethroids are generally considered to produce the T intoxication syndrome and the Type II pyrethroids are considered to produce the CS intoxication syndrome (Lawrence et al. 1982). The interchangeability between the Type I/II

nomenclature and T/CS nomenclature are useful as a general classification scheme and are generally used in the published literature. However, there are a number of pyrethroids that do not fit neatly into these schemes because there are Type I/II pyrethroids that can produce overlapping syndrome (T/CS) of intoxication. (Soderlund et al. 2002). Nonetheless, for the purposes of this thesis the reference to Type I and Type II pyrethroids represents the structural class and the pattern of intoxication syndrome of pyrethroids.

Hypothesis and Specific Aims

As a result of the evidence in literature (see above) showing that Type I and Type II pyrethroid insecticides target the VGSC and slows steady-state inactivation and that the literature showing (see above) that the β4 peptide mimics the open channel blocker that occludes the pore and leads to resurgent currents we formed a hypothesis that *β4 mediated resurgent currents in Nav1.5 VGSCs increase after exposure to Type I and Type II pyrethroid insecticides.*

This thesis highlights the effects of Type I and Type II pyrethroid insecticides on β4 peptide mediated Nav1.5 VGSC resurgent currents. The aims of this thesis were to first determine wild-type Nav1.5 channel activity and whether channel activity changes with exposure to the vehicle (DMSO) used to dilute insecticides. Results from the first aim, although not recorded in this thesis, demonstrated that DMSO at 0.1% has no significant effect on the wild-type Nav1.5 channel activity. Since this was the case, we moved forward with electrophysiological experiments using 0.1% DMSO as a vehicle in which we diluted 2μM and 10μM pyrethroid insecticides before exposing cells. The second aim investigated the β4 peptide's effect on these Nav1.5 VGSC currents and whether or not resurgent currents were produced. The results from the second aim demonstrated that in the presence of β4 peptide the Nav1.5 VGSC produces noticeable resurgent currents. The third aim investigated Nav1.5 channel

activity when exposed to Type I and II pyrethroid insecticides (Permethrin and Cypermethrin respectively). The results from the third aim demonstrated that exposure to the insecticides effects the rate of steady-state inactivation in Nav1.5 VGSCs as it produced significant tail and persistent currents. Finally, the fourth aim investigated β4 peptide mediated VGSC resurgent current activity after exposure to Type I and II pyrethroid insecticides. The results from the fourth aim demonstrated that β4 mediated resurgent currents increase after exposure to the pyrethroid insecticides.

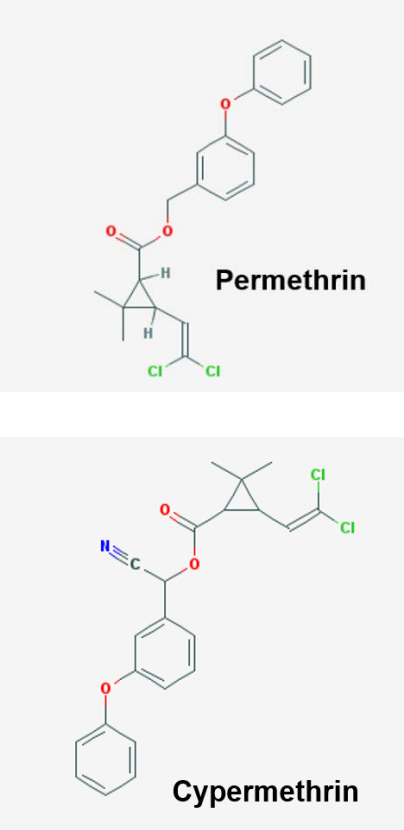

Figure 3. Chemical structure of Type I and Type II Pyrethroid Insecticides, Permethrin and Cypermethrin respectively. The two most commonly used pyrethroids in agriculture are Cypermethrin and Permethrin (Atchison et al., 2012).

Chapter Two: Materials and Methods

Preparation of Stably Transfected Cell Lines

Experiments investigating the effects of pyrethroid pesticides on β4 mediated resurgent currents in Nav1.5 channels were done using stably transfected cell lines. To make the stable cell line, Human embryonic kidney cells (HEK293 cells; American Type Culture Collection; Manassas, VA, U.S.A.) were used. They were incubated under typical tissue culture conditions (5% CO2; 37 ^{o}C) in complete Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Grand Island, NY, U.S.A.) supplemented with 10% fetal bovine serum (FBS; Cellgro, Herndon, VA, U.S.A) and 1% penicillin/streptomycin (Invitrogen Grand Island, NY, U.S.A.). The calcium phosphate precipitation method was used to perform transfections of Nav1.5 cDNA. The calcium phosphate precipitation method is as follows: solution 1 (40 µl 2X HEPES buffer) was added dropwise to solution 2 (4 µg of channel cDNA at 1µg/µl concentration and 5µl of 2M CaCl2; use sterile water to bring volume to 40µl) and was gently mixed using a pipette. The calcium phosphate-DNA mixture was incubated at room temperature for 30 minutes after which, it was added to HEK293 cells incubated in serum-free DMEM for one hour on a 100x20 mm culture petri dish. Following incubation for 12-24 hours at 37 ^{o}C, the cells were washed with fresh complete DMEM medium. The Nav1.5 cDNA was subcloned into the pcDNA 3.1 (+) vector using the HindIII Xbal restriction enzymes. This vector contains the neomycin (G418) resistant gene for the purpose of using G418 treatment to select for HEK cells stably expressing Nav1.5. After 48 hours of incubation in complete DMEM medium (10% FBS, 1% pen/strep) the antibiotic (G418, Geneticin; Cellgro, Herndon, VA) was added to the media to select for neomycin-resistant HEK293 cells. After 2-4 weeks in G418, HEK293 cell colonies resistant to G418 were isolated and allowed to grow on 12 mm glass coverslips (Microscope Cover Glass; Fisherbrand, Pittsburg, PA, U.S.A) coated in Laminin. Isolated cells from these colonies were tested for Nav1.5 channel

expression using whole-cell patch-clamp recording techniques. Colonies containing cells producing more than 1 nA of Nav1.5 current were used for subsequent experiments

Chemicals

Stock solutions of Permethrin (Sigma-Aldrich International, St. Louis, MO, U.S.A.) and Cypermethrin (Sigma-Aldrich International, St. Louis, Mo, U.S.A.) prepared in 0.1% Dimethylsulfoxide (DMSO, Sigma Aldrich International, St. Louis, Mo., U.S.A.) were aliquoted and stored at room temperature. For experiments, the insecticides were diluted in extracellular bathing solution to achieve a final concentration of 0.02 – 10µM in a final volume of 400µl in a micro centrifuge tube which was transferred, all 400µl by pipette, to the reservoir containing the cover slip with the stably transfected Nav1.5 HEK293 cells. After 10 minutes whole cell patch-clamp experiments were conducted.

The β4 peptide consisting of part of the C-terminal tail (KKLITFILKKTREK-OH) of the full-length β4 subunit was added to the intracellular pipette solution to induce Nav1.5 resurgent currents in stably transfected HEK293 cells. For this, stock solution of β4 peptide (Biopeptide Co., San Diego, CA) in water, (stored in -20^{o}C) was diluted in the intracellular pipette solution to achieve a final concentration of 200µM in a final volume of 500µl. This β4 peptide solution was used to back fill the recording pipette.

Solutions

1. Extracellular Bathing Solution

The standard extracellular bathing solution was used during all voltage clamp whole-cell electrophysiology experiments. The extracellular bathing solution containing (in mM): 140 NaCl, 1 MgCl2, 3 KCl, 1 CaCl2 and 10 HEPES, pH 7.3 (adjusted with NaOH), Osmolarity (280-300 mOsm, adjusted with NaCl) was measured using a Wescor VAPRO Vapor Pressure Osmometer 5520 (Logan, Ut, U.S.A). In experiments involving the pyrethroid pesticides effects on Nav1.5 kinetics

and β4 mediated resurgent currents, the pesticides were diluted to a final concentration of 0.02 – 10μM in extracellular bathing solution in a micro centrifuge tube, final volume of 400μl before being transferred by pipette to the reservoir containing the cover slip with the stably transfected Nav1.5 HEK 293 cells.

2. Intracellular Pipette Solution

The Intracellular pipette solution was used in all voltage clamp whole-cell electrophysiology experiments. The intracellular pipette solution containing (in mM): 140 CsF, 10NaCl, 1.1 EGTA and 10 HEPES, pH 7.3 (adjusted with CsOH), osmolarity 280-300 (adjusted with CsCl) was measured using a Wescor VAPRO Vapor Pressure Osmometer 5520 (Logan, Ut, U.S.A). The β4 peptide was diluted in pipette solution before being back-filled into the pipette.

Whole Cell Patch-Clamp Recordings

Whole cell patch-clamp recordings were conducted at room temperature (~ 21 degrees Celsius) using a HEKA patch clamp EPC 10 amplifier. A Windows-based Pentium IV computer using the Pulse program (v 8.78, HEKA Elektronik, Lambrecht/Pfalz, Germany) was used to acquire data. Fire-polished glass pipettes (measured at ~ 1 micron) were fashioned from 100ul Calibrated Pipets (Drummond Scientific Company, Broomall, PA, U.S.A) using a Sutter P-1000 Flaming/Brown Micropipette Puller (Novato, CA, U.S.A) and fire-polished using a Micro Forge MF-830 (Narishige, Japan). To hold coverslips containing stably transfected HEK293 cells during whole cell patch-clamp recordings, lids from 35 mm cell culture petri dishes (Corning Inc., Corning, NY, U.S.A) were filled with elastomer from 184 Silicone Elastomer Kit (Sylgard®, World precision Instruments Inc., Sarasota, FL, U.S.A.), mixed with a curing agent (included in kit), and allowed to solidify. Once solidified, a scalpel was used to cut around a single 12mm coverslip into the silicone to create a reservoir that could hold a coverslip and 400μl of extracellular solution.

The recording dish containing stably transfected HEK293 cells was mounted on an inverted Nikon microscope. The microscope was placed on a TMC vibration isolation table (63-500 Series, Technical Manufacturing Company, Peabody, MA, U.S.A.) to reduce all negative effects of vibration on the experiments. All whole cell patch-clamp electrophysiological experiments were conducted without the use of a Faraday cage. Instead, multiple wires were used to ground every metal surface that had the potential to serve as an antenna for unwanted electrical noise. These wires converged and connect at a single point on the isolation table; and from that point a single wire was connected to the ground input of the HEKA EPC 10 amplifier. Isolated, single cells on the cover slip were selected for whole cell patch-clamp electrophysiology. Each whole cell patch-clamp electrophysiological recording was collected using a new fire-polished pipette, back-filled with appropriate intracellular pipette solution. Once filled with the appropriate intracellular pipette solution, and once the pipette was submerged in the extracellular bathing solution in the dish, the recording electrode had an approximate resistance of 1.0 - 1.5 MΩ. The offset potential of the EPC 10 amplifier was zeroed with the electrode almost touching the cell of interest. A silver chloride coated silver wire served as a reference electrode with one end connected to the ground input of the amplifier head stage and the silver chloride coated end placed into the reservoir containing the coverslip with the stably transfected HEK 293 cells and extracellular bathing solution. The liquid junction potential for all solutions was not corrected for during these experiments and data analysis. Once a cell membrane-glass pipette interaction was formed, creating over 1GΩ resistance, suction was applied to establish the whole-cell recording configuration. All voltage protocols were started five minutes after entering the whole cell configuration, which allowed time for diffusion of the β4 peptide when experiments called for it. Series resistance errors were

compensated to be under 5mV using resistance compensation and passive leak currents were cancelled by P/-5 subtraction.

For all experiments, recordings were made in the presence of extracellular solution containing drug or vehicle control. Vehicle control was 0.1% DMSO final concentration in extracellular solution without the pyrethroid pesticice. Each coverslip was recorded from for up to one and half hours before discarding.

Data Analysis

All voltage-clamp electrophysiological recording experimental data were analyzed using the Pulsefit (v 8.65, HEKA Electronic, Germany), Prizm Graph-pad (7.03) and Microsoft Excel software programs. Normalized conductance – voltage relationships (G-V) were derived using the function: $G_{Na}=I_{Max}/(Vm-E_{Na})$. G_{Na} is conductance of sodium channel, I_{Max} is the peak current density in response to the test pulse, Vm stands for the test pulse potential, and E_{Na} is the measured sodium channel reversal potential. Slope factors of activation and steady-state inactivation curves were calculated using the general Boltzman function: $I/I_{max} = (1/1+e^{(V-V_{0.5})/k}$ where I is measured current, I_{max} is maximum current, V is command voltage, $V_{0.5}$ is voltage at which the normalized current value is 0.5, and k is slope factor describing the steepness of the relationship. Goodness of fit was set at $R^2 > 0.90$ for all fits. Results are presented as mean $\pm$ S.E.M., and error bars in the figures represent SEs. Statistical significance was set at $p < 0.05$ for all experiments.

Resurgent Current Analysis

Cells were assayed with a step protocol that initially depolarized the membrane to +30mV for 20ms from the holding potential, followed by repolarizing voltage steps from +15mV to -85 for 100ms in -5mV increments to test for resurgent currents; cells were then returned to their holding potential. Resurgent current amplitudes were measured from the leak-subtracted

baseline to the peak after 3.0ms into the repolarizing pulse to avoid contamination from tail currents. Relative resurgent currents were calculated by dividing peak resurgent current by the peak transient current and expressed as a percentage of the peak transient current. The peak transient current was determined as the peak from the h_∞ protocol. Student's t test was used to examine the statistical significance of relative resurgent current amplitude between groups.

Chapter Three: Results

Whole-cell voltage-clamp recordings were obtained from HEK293 cells stably expressing hNav1.5 channels (Nav1.5 cells). There was no observed significant change in the voltage-dependence of activation when Nav1.5 cells were exposed to Type I and Type II pyrethroid insecticides compared to control (see Figure 4). However, Type I and Type II pyrethroid insecticides did shift steady-state inactivation of Nav1.5 cells (see Figure 5). Cypermethrin shifted inactivation by ~5mV in the negative direction and Permethrin shifted Nav1.5 inactivation by ~5mV in the positive direction. However both insecticides resulted in incomplete inactivation as evidenced by the increased fraction available at voltages between -50mV and -10mV.

We also examined the rate of inactivation during depolarizations to 0 mV. In Nav1.5 cells we observed two components for the time course of fast-inactivation; a fast component and a slow component. Measuring the Tau time constants we found that Permethrin and Cypermethrin significantly increased the Tau for the slow inactivation component (see Figure 6). Cypermethrin had a much greater effect on the slow time constant but Permethrin also significantly slowed the fast time constant compared to control cells.

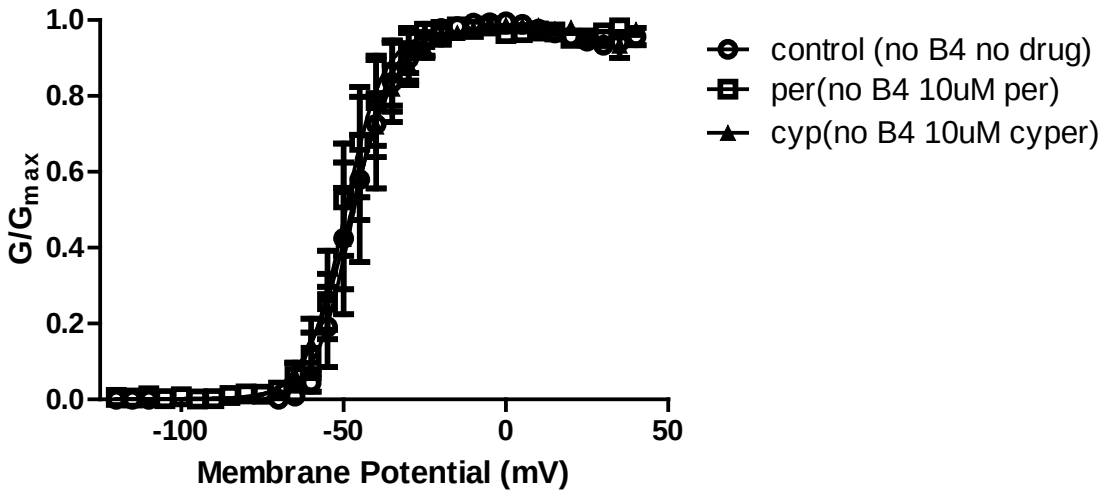

Figure 4. Effects of Permethrin and Cypermethrin on activation of sodium channel Nav 1.5.

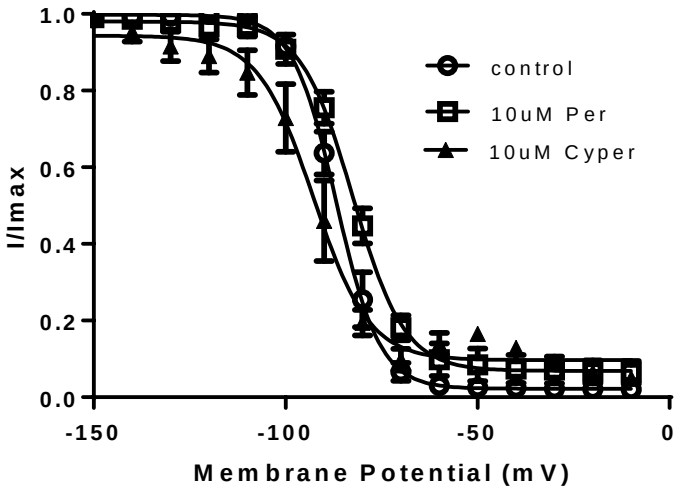

Figure 5. Effects of Permethrin and Cypermethrin on steady-state inactivation of sodium channel Nav1.5.

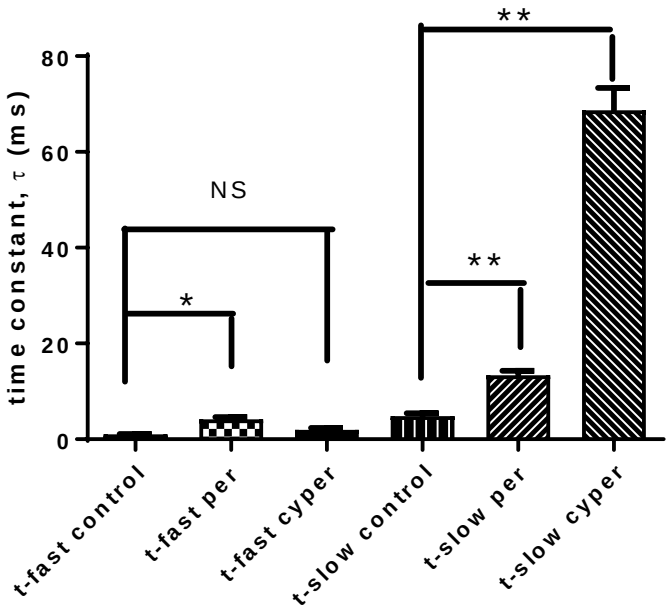

Figure 6. Effect of Permethrin and Cypermethrin on time constants of Nav1.5 inactivation.

Time constants were obtained by fitting double exponential functions. Student t-test was used to determine significance. NS, not significant; *, p-value < 0.05; **, p-value < 0.01.

We next examined sodium currents during repolarizing steps following a strong depolarization. A strong depolarization (20 ms at +30 mV) from the holding potential is followed by repolarizing voltage steps ranging from +15 mV to -85 mV for 100 ms in -5 mV increments. This protocol can be used to examine resurgent sodium currents in the presence of the β4 peptide. In the absence of the β4 peptide the control panel shows a complete inactivation with no evidence of tail currents or persistent currents during the repolarizing voltage potentials (see Figure 7). However, after exposure to Permethrin and Cypermethrin we see significant production of tail currents and persistent current during the repolarizing voltage potentials.
In the presence of the β4 peptide the control panel shows β4 mediated resurgent current during the repolarizing potentials. After exposure to Permethrin and Cypermethrin we see larger resurgent currents during the repolarizing potentials compared to control. We also see that Cypermethrin produces larger and slower resurgent current compared to the resurgent current that Permethrin produces. It appears that Permethrin and Cypermethrin have a synergistic effect and combined with the β4 peptide they increase the size of the resurgent currents.
It is important to note the repolarization induced currents differ in the absence and presence of the β4 peptide. In the absence of the β4 peptide, the insecticides increase persistent currents during the depolarization which leads to instantaneous classic “tail currents” during the repolarizing pulses. Because inactivation is virtually complete under control conditions, no tail currents are observed in the absence of insecticides (see Figure 7 control). By contrast in the presence of the β4 peptide resurgent currents with a delayed onset compared to tail currents are observed (see Figure 8). The resurgent currents are significantly larger than the tail currents (note the different scales in Figure 9). Cypermethrin induced resurgent currents are both larger and slower than control and Permethrin induced tail currents.

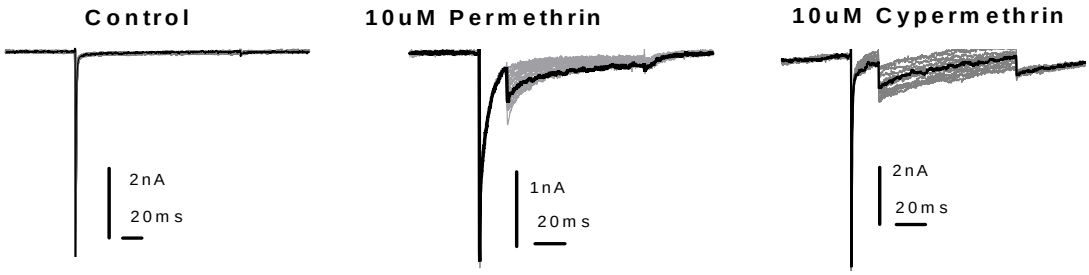

Figure 7. Representative trace of effects of Permethrin and Cypermethrin on Nav1.5 currents elicited by repolarizations. Both Permethrin and Cypermethrin increase, what appears to be persistent and tail current in Nav1.5 VGSCs.

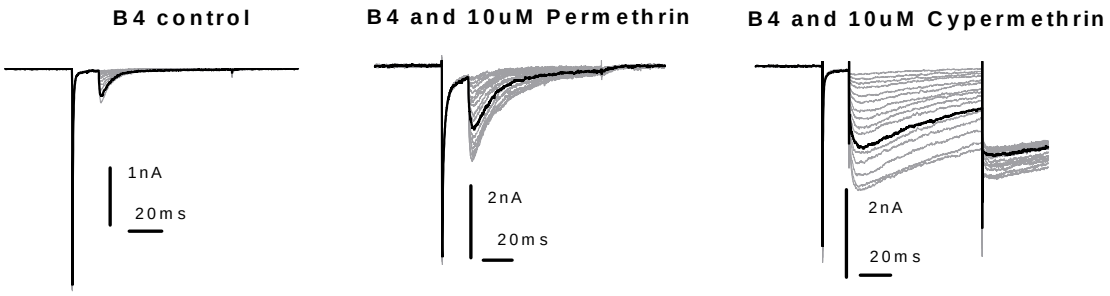

Figure 8. Representative traces illustrating the effects of Permethrin and Cypermethrin on β4 mediated resurgent currents. These traces show that the Type I and Type II pyrethroid insecticides act synergistically with the β4 peptide to increase resurgent currents in Nav1.5 after exposure of cells to Permethrin and Cypermethrin.

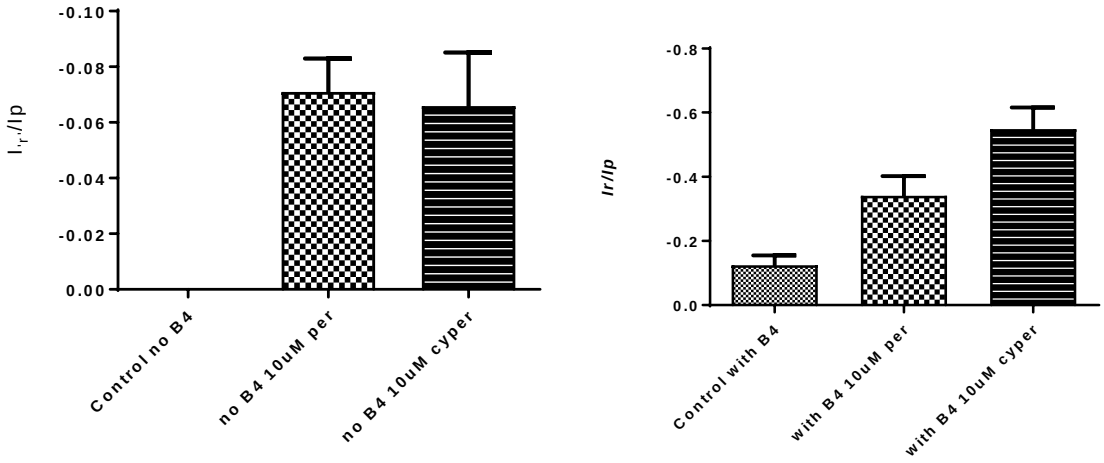

Figure 9. Repolarization induced current measurement in Nav1.5 cells before and after exposure to Permethrin and Cypermethrin. Permethrin and Cypermethrin produce significant repolarization-induced currents in Nav1.5 without β4 compared to the control. After Nav1.5 cells are exposed to Permethrin and Cypermethrin β4 mediated resurgent increase.

Chapter Four: Discussion

Results for this thesis demonstrate that 1) Nav1.5 channel activity does not change with exposure to the vehicle (DMSO) used to dilute Type I and Type II pyrethroids, data is not shown in the thesis; 2) the β4 peptide induces resurgent currents in Nav1.5; 3) Type I and Type II pyrethroids alters electrophysiological properties of Nav1.5 channel activity by slowing down steady-state inactivation; and 4) β4 peptide mediated VGSC resurgent current activity increases after exposure to Type I and II pyrethroid insecticides.

As mentioned above Permethrin is a Type I pyrethroid insecticide and Cypermethrin is a Type II pyrethroid insecticide. The major difference between Type I and Type II pyrethroids is that the Type II insecticide has an added α cyano moiety (see Figure 3). This added α cyano moiety changes Intoxication syndromes. Intoxication syndromes are different because the Type I and Type II pyrethroids keep channels open and keep them from inactivating but studies show that Type II pyrethroids keep sodium channels open longer than Type I pyrethroids (see above). We see this demonstrated in how much the B4 peptide mediated resurgent current exposed to the Cypermethrin is slowed down compared to control and the Permethrin panel (see Figure 8). Since this is the case, Cypermethrin would lead to more pronounced cardiac toxicity because the longer amount of time it keeps the channel open which will cause increased arrhythmias which can lead to death. As a result of these difference in the Type I and Type II pyrethroid insecticides and the different intoxication symptoms that are exhibited in mammals we could predict that these two pyrethroid insecticides would produce differences in cardiac toxicity.

Future direction for this project should include increasing the sample size of all of the conditions (i.e. control, Permethrin with or without β4, and Cypermethrin with or without β4) so that statistical significance can be determined more convincingly. Also, since it appears that there is a synergistic relationship between the pesticides and the β4 peptide in the production

of resurgent current it would be interesting to see the effects of both Permethrin and Cypermethrin with or without β4 protein on myocyte sodium channel activity. This will help determine the relationship, whether additive, synergistic or antagonistic, between the Type I and Type II pesticides and how the two of these affect the β4 peptide mediated resurgent current in native myocytes.

Testing the Type I and Type II pesticides with or without β4 peptide in myocytes from β4 peptide knockout mice would be an informative step forward. All experiments in this thesis were conducted in Nav1.5 stably transfected HEK293 cells. One of the main limitations in using heterologous expression systems is that they are an artificial system and since this is the case any physiological significance would have to be extrapolated from the data. It is more suitable to determine physiological significance in the primary tissues where Nav1.5 channels are localized, such as the myocytes. In myocytes we can first test to see if there are any endogenous β4 subunits using immunohistochemistry techniques. If β4 subunit is present we can test if it produces resurgent current using the resurgent current protocol. If it does produce resurgent currents we can see if the size of the resurgent currents are influenced by pesticide exposure. We can use selective knock-out β4 in myocytes and examine if toxicity of pyrethroid insecticides is reduced.

Literature shows that these pesticides function by slowing down the inactivation of sodium channels. It would be fitting to see how these pesticides combined with the effects of the endogenous β4 or β4 peptide affect neuronal resurgent currents. Due to the pyrethroid insecticide intoxication symptoms it would be important to determine if the effects of pesticides and β4 peptide on neuronal channels are similar to those observed with Nav1.5. Resurgent currents in neurons could lead to altered excitability including potentially to ataxia and seizures.

In conclusion, this thesis addressed key questions about how Type I and Type II pesticides affect β4 mediated resurgent currents in Nav1.5 cells. The results of this thesis have provided important insights into how the Type I and Type II pesticides modulate the Nav1.5 channel kinetics and how resurgent currents are modulated after exposure to the pesticides.

References List

1. Armstrong CM, and Bezanilla F. *Charge movement associated with the opening and closing of the activation gates of the Na channels.* J Gen Physiol., 1974. 63(5): 533-552.
2. Atchison WD, et al. *Pyrethroids and their effects on Ion Channels*. INTECH, 2012. 39-66.
3. Bant, JS and Raman, IM. *Control of transient, resurgent, and persistent current by open-channel block by Na channel beta4 in cultured cerebellar granule neurons.* Proc Natl Acad Sci., 2010. 107(27): 12357-62.
4. Barnes JM, Verschoyle RD. *Toxicity of new pyrethroid insecticide.* Nature, 1974. 248:711.
5. Barbosa C, et al. *Navbeta4 regulates fast resurgent sodium currents and excitability in sensory neurons.* Mol Pain, 2015. 11:60.
6. Bennett PB, Valenzuela C, Chen LQ, Kallen RG. On the molecular nature of the lidocaine receptor of cardiac Na channels. Modification of block by alterations in the α-subunit III-IV interdomain. Circ Res. 1995; 77: 584-592.
7. Casida JE. *Pyrethrum flowers and pyrethroid insecticides.* Environ Health Perspective, 1980. 30:189-202.
8. Catterall WA. *From ionic currents to molecular mechanisms: the structure and function of voltage-gated sodium channels.* Neuron, 2000. 26(1):13-25.
9. Cohen SA. *Immunocytochemical localization of rH1 sodium channel in adult rat heart atria and ventricle. Presence in terminal intercalated disks.* Circulation, 1996. 94: 3083-3086.
10. Costa, L.G. Basic Toxicology of Pesticides. In M. C. Keifer, M.D., M.P.H. (Ed.), *Human Health Effects of Pesticides.* Occupational Medicine. State of the Art Reviews, 1997. (Vol. 12 No. 2). (pp.251-268). Philadelphia: Hanley & Belfus, Inc.
11. Elliott, M. *The pyrethroids: early discovery, recent advances and the future.* Pestic. Sci., 1989. 27: 337-351.
12. Gammon DW, Brown MA, Casida JE. *Two classes of pyrethroid action in the cockroach.* Pestic Biochem Physiol., 1981. 15:181-191
13. Goldin AL. *Resurgence of sodium channel research.* Annu Rev Physiol., 2001. 63:871-894.
14. Goldin AL. *Evolution of voltage-gated Na(+) channels.* J Exp Biol., 2002. 205:575-584.
15. Greico, TM, et al., *Open-channel block by the cytoplasmic tail of sodium channel beta4 as a mechanism for resurgent sodium current*. Neuron, 2005. 45(2): 233-44.
16. Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ. *Improved patch clamp techniques for high-resolution current recording form cells and cell-free membrane patches.* Pflugers Arch., 1981. 391:85-100.
17. Hille B. *Pharmacological modifications of the sodium channels of frog nerve.* J Gen Physiol., 1968. 51:199-219.
18. Hodgkin AL, Huxley AF. *Currents carried by sodium and potassium ions through the membrane of the giant axon of Loligo*. J Physol., 1952(a). 116:449-472.
19. Hodgkin AL, Huxley AF. *The components of membrane conductance in the giant axon of Loligo.* J Physiol, 1952(b) 116:473-496.
20. Jarecki, BW, et al. *Human voltage-gated sodium channel mutations that cause inherited neuronal and muscle channelopathies increase resurgent sodium currents*. Journal of Clinical Investigation, 2010. 120(1): 369-78.
21. Kallen RG, Sheng ZH, Yang J, Chen LQ, Rogart RB and Barchi RL. *Primary structure and expression of a sodium channel characteristic of denervated and immature rat skeletal muscle.* Neuron, 1990. 4: 233-242.

22. Khaliq ZM, Gouwens NW, and Raman IM. *The contribution of resurgent sodium current to high-frequency firing in Purkinje neurons: an experimental and modeling study*. J Neuroscience, 2003. 23(12): p. 4899-912.
23. Lawrence LJ, Casida JE. *Pyrethroid toxicology: mouse intracerebral structure-toxicity relationships.* Pestic Biochem Physiol., 1982. 18:9-14.
24. Lee SH, Soderlund DM. *The V410M mutation associated with pyrethroid resistance in Heliothis virescens reduces the pyrethroid sensitivity of house fly sodium channels expressed in Xenopus oocytes Insect.* Biochem Mol Biol., 2001. 31:19-29.
25. Miyazaki H, et al., *Singular localization of sodium channel beta4 subunit in unmyelinated fibres and its role in the striatum*. Nat Commun., 2014. 5: 5525.
26. Moorman JR, Kirsch GE, Brown AM, Joho RH. *Changes in sodium channel gating produced by point mutations in a cytoplasmic linker.* Science, 1990. 250(4981):688-691.
27. Naeher LP, Tulve NS, Egeghy PP, Barr DB, Adetona O, Fortmann RC, Needhan LL, Bozeman E, Hilliard A, Sheldon LS. *Organophosphorus and pyrethroid insecticide urinary metabolite concentrations in young children living in a southeastern United States city.* Sci Total Environ., 2010. 408:1145-1153.
28. Narahashi T, Moore JW, Scott WR. *Tetrodotoxin blockage of sodium conductance increase in lobster giant axons.* J Gen Physiol., 1964. 47:965-974.
29. Narahashi T, Hass HG, Therrien EF. *Saxitoxin and tetrodotoxin comparison of nerve blocking mechanism.* Science, 1967. 157:1441-1442.
30. Narahashi T. *Neuronal ion channels as the target sites of insecticides.* Pharmacol Toxicol., 1996. 78:1-14.
31. Noda M, Shimizu S, Tanabe T, Takai T, Kayano T, Ikeda T, Takahashi H, Nakayama H, Kanaoka Y, Minamino N. *Primary structure of electrophorus electricus sodium channel deduced from cDNA sequence.* Nature, 1984. 312:121-127.
32. Nutter TJ, et al. *Persistent Na^+ and K^+ channel dysfunctions after chronic exposure to insecticides and Pyridostigmine bromide.* NeuroToxicology, 2013. 39: 72-83.
33. Pickett, JA. *New Opportunities in neuroscience, but a great danger that some may be lost.* In: Beadle DJ, Mellor IR, Usherwood PNR, editors. Neurotox '03: Neurotoxicological targets from functional genomics and proteomics. Society of Chemical Industry; London: 2004. 1-10.
34. Power LE, Sudakin DL. *Pyrethrin and pyrethroid exposures in the United States: A longitudinal analysis of incidents reported to poison centers*. Journal of Medical Toxicology, 2007. 3:94-99.
35. Raman IM, Bean BP. *Resurgent sodium current and action potential formation in dissociated cerebellar Purkinje neurons*. J Neurosci, 1997. 17(12): 4517-26.
36. Raman IM, Bean BP. *Ionic currents underlying spontaneous action potentials in isolated cerebellar Purkinje neurons*. J Neuroscience, 1999. 19(5): 1663-74.
37. Raman IM, Bean BP. *Inactivation and recovery of sodium currents in cerebellar Purkinje neurons: evidence for two mechanisms*. Biophs J., 2001. 80(2): 729-37.
38. Ranson H, N'Guessan R, Lines J, Moiroux N, Nkuni Z, Corbel V. *Pyrethroid resistance in African anopheles mosquitoes: what are the implications for malaria control?* Trends Parasitol., 2011. 27:91-98.
39. Ray DE and Forshaw PJ. *Pyrethroid insecticides: poisoning syndromes, synergies and therapy.* J. Toxicol. Clinl Toxicol. 2000. 38:95-101.
40. Ray DE. *Pyrethroid insecticides: mechanisms of toxicity systemic poisoning syndromes, paresthesia, and therapy.* In: Handbook of Pesticide Toxicology: Vol 2: Aents (Krieger R, Doull J, Ecobichon D, eds). San Diego: Academic Press, 2001. 1289-1303.

41. Rogart RB, Cribbs LL, Muglia LK, Kephart DD, Kaiser MW. *Molecular cloning of a putative tetrodocoxin-resistant rat heart Na+ channel isoform.* Proceeding of the National Academy of Sciences of the United States of America, 1989. 86(20):8170-8174.
42. Sackmann B, Neher E. *Patch clamp techniques for studying ionic channels in excitable membrane.* Annu Rev Physiol, 1984. 46:455-472.
43. Smith TJ, Soderlund DM. *Action of the pyrethroid insecticide cypermethrin on rat brain lla sodium channels expressed Xenopus oocytes.* Neuroticicology, 1998. 19:823-832.
44. Smith TJ, Soderlund DM. *Potent actions of the pyrethroid insecticides cismethrin and cypermethrin on rat tetrodotoxin-resistant peripheral nerve (SNS/PN3) sodium channels expressed in Xenopus oocytes.* Pestic Biochem Physiol, 2001. 70:52-61.
45. Soderlund DM, Bloomquist JR. *Neurotoxic actions of pyrethroid insecticides.* Annu. Rev, Entomol, 1989. 34, 77-96.
46. Soderlund DM. *Molecular Mechanisms of Pyrethroid Insecticide Neurotoxicity: Recent Advances.* Arch Toxicol., 2012. 86(2): 165-181.
47. Soderlund DM. *State-Dependent Modification of Voltage-Gated Sodium Channels by Pyrethroids.* Pestic Biochem Physiol., 2010. 97(2): 78-86.
48. Soderlund DM, Clark JM, Sheets LP, Mullin LS, Piccirillo VJ, Sargent D, Stevens JT, Weiner ML. *Mechanisms of pyrethroid toxicity: implications for cumulative risk assessment.* Toxicology, 2002. 171:3-59.
49. Spencer CI, Yuill KH, et al. *Actions of Pyrethroid Insecticides on Sodium Currents, Action Potentials, and Contractile Rhythm in Isolated Mammalian Ventricular Myocytes and Perfused Hearts.* Journal of Pharmacology and Experimental Therapeutics, 2001. 298: 1067-1082.
50. Spencer CI, et al. *Mechanisms Underlying the Effects of the Pyrethroid Tefluthrin on Action Potential Duration in Isolated Rat Ventricular Myocytes.* Journal of Pharmacology and Experimental Therapeutics, 2005. 315: 16-23.
51. Tan ZY, et al. *Tetrodotoxin-Resistant sodium channels in sensory neurons generate slow resurgent currents that are enhanced by inflammatory mediators*. Journal of Neuroscience, 2014. 34(21): 7190-97.
52. Theile JW, and Cummins TR. *Inhibition of Navbeta4 peptide-mediated resurgent sodium currents in Nav1.7 channels by carbamazepine, riluzole, and anandamide*. Mol Pharmacol., 2011. 80(4): 724-34.
53. Thatheyus AJ, Selvam, AG. *Synthetic pyrethroids: toxicity and biodegradation.* Applied Eco and Environ Sci., 2013. 1:33-36.
54. Tomlin C. (Ed.). *A World Compendium. The Pesticides Manual. Incorporating the agrochemicals handbook* (10th ed.). Bungay, Suffolk, U.k.: Crop Protection Publications, 1994.
55. Trainer VL, McPhee JC, Boutelet-Bochan H, Baker C, Scheuer T, Babin D, et al. *High affinity binding of pyrethroids to the alpha subunit of brain sodium channels.* Mol Pharmacol., 1997. 51:651-657.
56. Trimmer JS, Cooperman SS, Tomiko SA, Zhou JY, Crean SM, Boyle MB, Kallen RG, Sheng ZH, Barchi RL, Sigworth FJ, et al. *Primary structure and functional expression of a mammalian skeletal muscle sodium channel.* Neuron, 1989. 3(1):33-49.
57. Trimmer JS, Rhodes KJ. *Localization of voltage-gated ion channels in mammalian brain.* Annu Rev Physiol., 2004. 66:477-519.
58. Verschoyle RD, Barnes JM. *Toxicity of natural and synthetic pyrethrins to rats.* Pestic Biochem Physiol., 1972. 2:308-311.

59. Verschoyle RD, Aldridge WN. *Structure-activity relationships of some pyrethroids in rats.* Arch Toxicol., 1980. 45:325-329.
60. Vilin YY, Makita N, George AL, Jr., Ruben PC. *Structural determinants of slow inactivation in human cardiac and skeletal muscle sodium channels.* Biophys J., 1999. 77:1384-1393.
61. Want SY, Barile M, Wang GK. *A phenylalanine residue at segment D3-S6 in Nav1.4 voltage-gated Na^+ channels is critical for pyrethroid action.* Mol Pharmaclo., 2001. 60:620-628.
62. West JW, Patton DE, Scheuer T, Wang Y, Goldin AL, Catterall WA. *A cluster of hydrophobic amino acid residues required for fast Na(+)-channel inactivation.* Proc Natl Acad Sci USA, 1992. 89:10910-10914.
63. Yu FH and Caterall WA. *Overview of the voltage-gated sodium channel family.* Genome Biol., 2003. 4:207.

Curriculum Vitae

James O. Jackson, II

Education

- Master of Science in Pharmacology – August 2018
  Indiana University, Indianapolis, Indiana

- Master of Divinity (*Cum Laude*) – May 2012
  Christian Theological Seminary, Indianapolis, Indiana

- Master of Arts in Mental Health Counseling (*Cum Laude*) – May 2012
  Christian Theological Seminary, Indianapolis, Indiana

- Clinical Pastoral Education, Level I & II – May 2013
  Indiana University Health Downtown, Indianapolis, Indiana

- Bachelor of Science, Agronomy – May 2000
  North Carolina State University, Raleigh, North Carolina

- Bachelor of Science, Biological Science – December 1999
  North Carolina State University, Raleigh, North Carolina

Publications

1) Xiao Y, Jackson JO 2nd, Liang S, Cummins TR. Common molecular determinants of tarantula huwentoxin-IV inhibition of Na+ channel voltage sensors in domains II and IV. J Biol Chem. 2011 Jun 9; 286(31):27301-10.

2) Jarecki BW, Piekarz AD, Jackson JO 2nd, Cummins TR. Human voltage-gated sodium channel mutations that cause inherited neuronal and muscle channelopathies increase resurgent sodium currents. J Clin Invest. 2010 Jan; 120(1):369-78.

3) Xiao Y, Blumenthal K, Jackson JO 2nd, Liang S, Cummins TR. The tarantula toxins Pro TX-II and Huwentoxin-IV differentially interact with human Nav 1.7 voltage sensors to inhibit channel activation and inactivation. Mol Pharmacol. 2010 Dec; 78(6):1124-34.

4) Jarecki BW, Sheets PL, Xiao Y, Jackson JO 2nd, Cummins TR. Alternative splicing of Na(V)1.7 exon 5 increases the impact of the painful PEPD mutant channel I1461T. Channels. 2009 Jul-Aug; 3(4):259-67.

5) Jarecki BW, Sheets PL, Jackson JO 2nd, Cummins TR. Paroxysmal extreme pain disorder mutations within the D3/S4-S5 linker of Nav1.7 cause moderate destabilization of fast inactivation. J Physiol. 2008 Sep 1;586(Pt 17):4137-53.

6) Veronesi MC, Yard M, Jackson J, Lahiri DK, Kubek MJ. An analog of thyrotropin-releasing hormone (TRH) is neuroprotective against glutamate-induced toxicity in fetal rat hippocampal neurons invitro. Brain Res. 2007 jan 12;1128(1):79-85.

7) Sheets PL, Jackson JO 2nd, Waxman SG, Dib-Hajj SD, Cummins TR. A Nav1.7 channel mutation associated with hereditary erythromelalgia contributes to neuronal hyperexcitability and displays reduced Lidocaine sensitivity. J Physiol. 2007 Jun 15;581(Pt 3):1019-31.

Skills

- Molecular biology, biochemistry, and cell biology techniques such as PCR, Gel Electrophoresis, Western Blots, Site directed mutagenesis, Immunocytochemistry, Flow Cytometry, Transfection protocols, Cell culture, Tissue culture, Good Laboratory Practice, DNA/RNA isolation, Microscopy, Fluorescent Microscopy, Patch clamp techniques, Whole cell electrophysiology, Protein extraction, Protein assays, Cryostat sectioning, Small animal handling, Dissections, Survival surgeries

- Public speaking, Grief counseling, Conflict management, Mediation, Crisis management, Marriage and family counseling, Mental health counseling, Community outreach, Teaching, Training, Mentoring, Coaching, Chaplaincy, Event planning

Professional Laboratory Experience

Operations Director
IU School of Medicine, Indianapolis, Indiana
Neurosciences Research Building, Stark Neurosciences Research Institute
2014 – Present
- Manage the day-to-day operations of the Neuroscience Research Building (i.e., maintenance, training, inventory, ordering)
- Maintain research regulatory paper work (IBC protocols and IACUC protocols) and ensure that all NB labs meet all biosafety, government, and institutional requirements and that all personnel are in compliance
- Conduct monthly meetings with Lab Managers for training updates and user compliance

Core Lab Manager
IU School of Medicine, Indianapolis, Indiana
Neurosciences Research Building, Stark Neurosciences Research Institute
2009 – Present
- Oversee the day-to-day operations of the Core Lab facility; product inventory, purchasing; coordinating facility activities; scheduling lab space and equipment for ongoing projects; liaison for inter/intra departmental/institutional cooperation and collaborations, maintain and repair equipment, schedule preventative maintenance, and negotiate service contracts
- Maintain research regulatory paper work (IBC protocols and IACUC protocols) and ensure that the Core Lab facilities meet all biosafety, governmental, and institutional safety requirements and that all personnel are in compliance
- Discuss experimental needs, technical issues and new equipment acquisitions with Director, Principle Investigators, postdoctoral fellows, graduate students, and lab technicians and implement solutions
- Conduct monthly meetings with lower level technicians, lab managers, graduate students, and post-doctoral students for training updates and Core Lab user compliance

Lab Manager (Theodore Cummins Lab)
IU School of Medicine, Indianapolis, Indiana
Stark Neurosciences Research Institute
2004 – 2014

- Manage the day-to-day operations of the lab which include chemical and product inventory, ordering, training, supervising lab personnel, and monitoring progress of projects
- Maintain research regulatory paper work (IBC protocols and IACUC protocols) and ensure that the lab meets all biosafety, government, and institutional requirements and that all personnel are in compliance
- Animal handling, tissue dissections and surgeries

Research Associate Scientist
Roche Diagnostic, Fishers, Indiana
2003 – 2004

- Perform basic and specialized testing of research and preclinical hematology analysis
- Process samples and prepares blood samples for testing developing Glucose monitoring technology
- Independently operate, calibrate, maintain, and troubleshoot routine and specialized analytical instruments and equipment

Research Technician I
IU School of Medicine, Indianapolis, Indiana
Herman B. Wells Center for Pediatric Research, Indianapolis, Indiana
2001 – 2003

- Plan and conduct experiments related to DNA repair proteins as therapeutic cancer treatment
- Stay up to date with scientific literature, and methods that are pertinent to my project
- Animal handling, dissections and surgeries
- Train undergraduate work-studies, graduate students and post docs on research techniques, research protocols and methods

Field Biologist – Seeds & Traits R&D
Dow AgroSciences, Zionsville, Indiana
2000 – 2001

- Coordinate and perform functions related to Greenhouse management and corn breeding including, planting, gene checking, pollinating, seed packaging, plot maintenance, tissue sampling, note taking, harvesting and data entry
- Conduct high throughput nucleic acid extraction using fully automated and semi-automated protocols
- Utilize, monitor and maintain liquid handling robots, and vision-imaging systems

Lab Assistant
N.C. State University, Raleigh, North Carolina
USDA/ARS Plant Science Research Unit
1997 – 2000

- Assist Principle Investigator in conducting air pollution research in corn and soybean plants

- Coordinate and perform functions related to Greenhouse management: planting, gene checking, pollinating, watering, fertilizing, seed packaging, plot maintenance, tissue sampling, note taking, harvesting and data entry

Lab Assistant
N.C. State University, Raleigh, North Carolina
Department of Zoology
1996 – 1997

- Assist Principle Investigator in conducting animal behavior research in salamanders and fish
- Cryostat Sectioning; thin – section fixing and staining
- Animal handling, dissections and surgeries

Military Service

United States Army Reserves, 310th ESC, Fort Benjamin Harrison, Indiana, 1st Lieutenant, Chaplain Section, March 2009 – December 2013
United States Army Reserves, 310th ESC, Fort Benjamin Harrison, Indiana, 2nd Lieutenant, Chaplain Section, August 2007 – March 2009