Acknowledgements

Dr. French, thank you for your guidance and support throughout this project. Even with the circumstances I never felt unprepared or overwhelmed because of your assistance. Also, I cannot thank you enough for writing a recommendation letter for graduate school! I can honestly say that I have learned lessons over the course of this project about patience, dedication, and what it means to put everything I have towards a goal. Again I cannot thank you enough for all of your help and encouragement.

Dr. Hersh, during Junior Seminar I can tell you that our proposals were intense, and we were kept quite busy! Although there was a lot to do I felt very prepared for the brand new proposal I had to submit for my senior project. Also thank you for your willingness to talk about everything and your comments on my drafts.

Justin Gill, thank you for your help with dividing up common tedious labor. I cannot tell you how much easier working with you made everything. From changing solutions and holding each other accountable to reading over drafts and talking about what we REALLY did during our procedures everything went so much smoother over the course of this project because of your help.

To my family, thank you for all of your love and support throughout the years. From the beginning of my days in school to graduation from college and beyond I cannot tell you how appreciative I am for your encouragement to do my best and to achieve whatever it is I want to achieve. Mom and Dad, as I have mentioned before I can only hope to become as loving and caring of a parent as you both are. Who I am and what I will become is a direct reflection of how you have raised me and your influence on me personally. Stephen, thank you for always sitting around and having brother time with me when I come home from school. I know that you will achieve great things one day no matter what you choose to do with your future.

Abstract

The pharmaceutical industry has been searching for a specific T-Type calcium channel blocker for decades. The development of ziconotide, which was derived from an omega conotoxin, as a treatment for chronic pain led to an upsurge of research centered on conotoxins as potential therapeutic agents. Conotoxins are neurotoxic peptides from *Conus* venom. Conus snails use their venom to paralyze their prey by targeting its ion channels. The scope of the *Conus* venom has been estimated to contain more than 50,000 different pharmacologically active components, with 50 to 200 different active components per species. It has been widely reported that the most potent venom is utilized by *Conus geographus*, a piscivorous marine cone snail known to cause human fatalities. This study was designed to analyze the effects of *C. geographus* venom over time on $Ca_v3.2$ calcium channels expressed in *Xenopus laevis* oocytes using two-electrode voltage clamp techniques. The results of this study indicate that *C. geographus* crude venom inhibits $Ca_v3.2$ calcium channels. Moreover, the venom must contain a conotoxin that selectively targets naturally occurring $Ca_v3.2$ calcium channels. Identifying this conotoxin could provide the pharmaceutical industry with an effective treatment for epilepsy, cardiac hypertrophy, cancer, chronic pain, and other serious health concerns.

Introduction

The properties of currents mediated by ion channels were first published by Hodgkin and Huxley in 1952. This seminal research paper provided the mathematical models used as the basis for understanding ion channels studied today. Since then, different ion channels have been widely reported as controllers of muscle action, central nervous system functioning, and many other body processes (Fatt and Katz 1953; Huxley 2002). Ion channels are proteins that allow transport of charged molecules across a cellular membrane. Transport occurs through a pore-forming subunit that is gated by two general types of stimuli: ligands or voltage (Hille 2001). Ligand-gated channels are opened or closed by chemical messengers, like neurotransmitters, that bind to channels and change their conformation (Purves et al. 2007). Voltage-gated ion channels, on the other hand, are activated and deactivated by changes in membrane potential ($Voltage_{INTERIOR}$-$Voltage_{EXTERIOR}$) (Dolphin 2006).

Voltage-gated ion channels can move sodium, calcium, potassium, and other ions across the cellular membrane in response to a localized electrical current. However, ion channels are selective in which type of ions they move across the cellular membrane. Often they can transport one main type of ion due to size and charge constraints (Bear et al. 2002).

One type of voltage-gated ion channel, the calcium channel, can be further subdivided into high voltage- (HVA), intermediate voltage- (IVA), and low voltage-activated (LVA) variations (Catterall et al. 2005). All three variations are composed of four to five different subunits, each encoded by separate genes, that determine channel function (Catterall 2000). The α-1 subunit contains the conduction pore, voltage sensor, gating component, and the majority of receptor sites for second messengers, drugs, and toxins. α-1 subunits are

comprised of four homologous domains (I-IV) with each domain containing six transmembrane segments (S1-S6) (Fig 1). The S4 loop recognizes changes in voltage across the membrane and signals those changes to the rest of the domain. Between the S5 and S6 segments there is a pore loop that dictates ion selectivity and conductance (Dirkson et al. 1997; Hofmann et al. 1994; Nichols 1994). While the α-1 subunit is the most essential of all the subunits, the others (β, γ, α2δ) have been tied to channel activation rates and positioning of the channels throughout the membrane (Hofmann et al. 1999; Yunker and McEnery 2003).

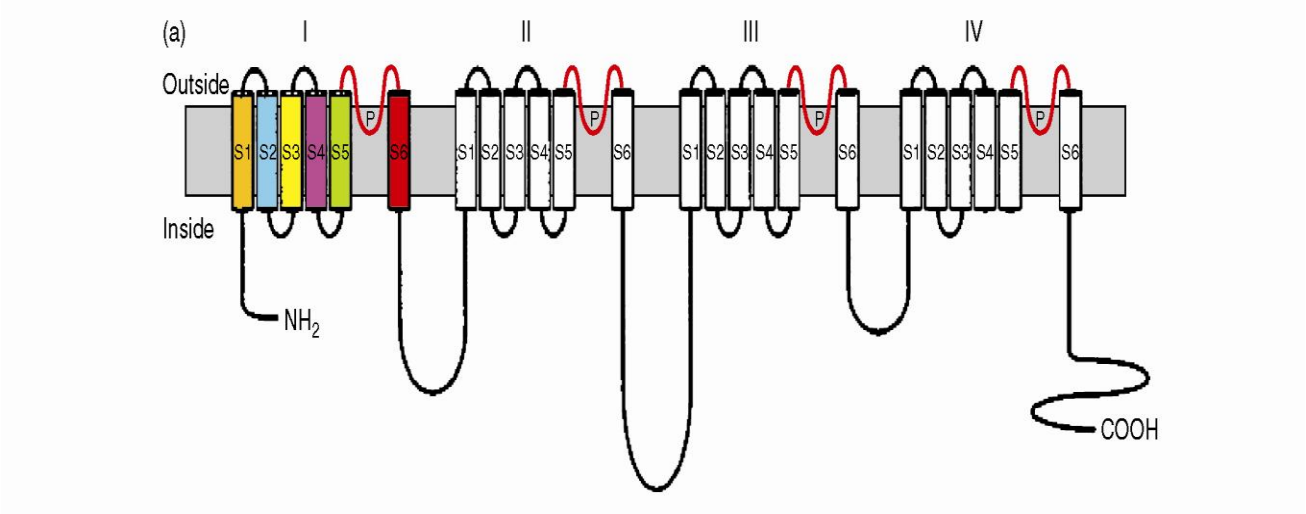

Figure 1: The general α-1 subunit of voltage sensitive calcium channels. The subunit contains four homologous domains (I-IV) that contain six transmembrane segments each (S1-S6). Between S5 and S6 there is a pore loop that dictates ion flow through the pore (Hammond 2008).

Although all contain a similar α-1 subunit, the three variations of voltage-sensitive calcium channels differ greatly. HVA channels are comprised of three different types of channels: L, P, and N. L-Type channels are found in the heart as well as smooth and skeletal muscles. Their function is to help regulate cell excitation and muscle contraction. P- and N-Type channels are located in nerve terminals and dendrites, and both have a role in neurotransmitter release (Catterall et al. 2005; Spedding and Paoletti 1992). IVA, or R-Type, channels are located in cerebellar granule cells, but their function is poorly understood

(Raybaud A 2007). LVA, or T-Type, channels differ greatly from HVA and IVA calcium channels.

The main difference between LVA channels and HVA or IVA variations is that LVA channels have an activation threshold of -70 to -40 mV whereas HVA and IVA channels require more depolarized voltages to activate (Vassort and Alvarez 1994). LVA channels can contain one of three α-1 pore-forming subunits that dictate their activation: $Ca_v3.1$, $Ca_v3.2$, or $Ca_v3.3$. The first of these three, $Ca_v3.1$ subunits, are activated at voltages around -46 mV and are found largely in the brain (Monteil A 2000; Perez-Reyes 1998). The second, $Ca_v3.2$ subunits, are also most active around -46 mV, but are found in more locations throughout the human body including smooth and skeletal muscle. The third type of subunit, $Ca_v3.3$, activates at voltages close to -44 mV and is located mostly in the brain (Catterall et al. 2005; Klöckner, 1999).

$Ca_v3.1$ channels have been shown to be the most evolutionarily conserved of the three channels across different species. As a result, $Ca_v3.2$ and $Ca_v3.3$ channels have been suggested as the cause of T-Type calcium channel variation between organisms (Perez-Reyes 2006). $Ca_v3.2$ channels are defined by a few main characteristics similar to those of $Ca_v3.1$. Similarities to $Ca_v3.1$ include inactivation at low voltages and slow deactivation (Kim et al. 2006). Although $Ca_v3.2$ channels are similar to $Ca_v3.1$, the two can be differentiated by their rates of recovery from inactivation, sensitivity to nickel blocks, and the presence of an α1-H pore forming subunit in the $Ca_v3.2$ channels as opposed to an α1-H subunit in $Ca_v3.1$ (Perez-Reyes 2006).

T-Type calcium channels have been found to mediate a plethora of important body processes including neuronal firing. Since T-Type channels can respond to small positive

changes in membrane potential, they can create a window current. A window current, the small range of voltages where T-type channels can open, but do not inactivate completely, allows for an initial inward of calcium ions close to the resting membrane potential of a cell. Window currents have been suggested as the source of varying neuronal firing patterns that result in distinct network rhythms. These distinct network firing rhythms allow T-Type channels to control pacemaker activity, which is the creation of rhythmic impulses to control a heartbeat, in the heart as well as low-threshold spikes that foster pro-epileptic and sleep-related "burst-firing" (Perez-Reyes 2006; Snutch and Cain 2010).

T-Type channels also have a role in neuropathic pain. Peripheral nociceptors are integral to pain-response and are part of the dorsal root ganglia. $Ca_v3.2$ channels in particular are highly expressed in the dorsal root ganglia, and have been tied to the control of ionic currents that contribute to the excitability of peripheral nociceptors. Neuropathic pain disorders, like Fibromyalgia, stem from damage to the peripheral nervous system. Insofar as it can be ascertained, $Ca_v3.2$ channels play a pivotal role in the causality of such disorders to the peripheral nervous system, and as a result they can affect neuropathic pain. Pharmaceutical treatments for neuropathic pain are limited, and the use of current treatments can lead to a copious amount of side-effects. A compound that could target $Ca_v3.2$ channels exclusively may be the solution to finding a treatment without serious side-effects (Hildebrand et al. 2010).

Unfortunately for the drug industry and ion channel researchers, very few compounds have been identified and characterized as selective T-Type calcium channel modifiers. Potential modifiers may exist in cone snail venom (Genus: *Conus*). The *Conus* genus encompasses a variety of predatory snails that utilize complex, peptide-rich, venoms as an

attack mechanism to paralyze their prey. Each of the 500 species in the *Conus* genus has its own unique venom that it uses to attack prey. The different species can be divided into three main groups based on the type of prey they target. These three groups include vermivorious (worm-hunting), molluscivorous (snail-hunting), and piscivorous (fish-hunting) snails. When attacking their prey, cone-snails use a harpoon-like tooth as a hypodermic needle to inject venom into the body of their target. The venom then rapidly paralyzes the prey and makes it an easy meal for the slow moving snails (Terlau and Olivera 2004).

It has been estimated that the scope of *Conus* venom includes greater than 50,000 different pharmacologically active components with 50 to 200 different active components in each species (Terlau and Olivera 2004). The *Conus* venom employs spectacular specificity and diversity in its selection of ion channel targets. It can selectively attack sodium, potassium, and calcium channels with a variety of different peptides called conotoxins that comprise its venom. Conotoxins can be divided into two main categories. One category includes conotoxins that have few (0 or 1) disulfide bridges and the other is composed of conotoxins that have two to five disulfide bridges. Disulfide bridges are important in the composition of the conotoxin because they provide structural rigidity to the peptide. The two categories of conotoxins are developed from seven different precursor genes (A, T, O, M, P, I, and S) (Luo et. al 2006).

Conotoxins have been shown to have a wide variety of pharmacological applications. To target their prey, the snails utilize their wide array of pharmacologically active components to attack many different ion channels in their target. One characteristic of the venom is the aforementioned specificity. The ability to specifically target ion channels is something that is desired by drug developers looking for remedies with minimum side

effects. Specific targets of the cone snails include neurotransmitters (Terlau and Olivera 2004). As it has been alluded to previously, neurotransmitters are very important in pain response and muscular function. Conotoxin targeting of neurotransmitter release led to the identification of an omega conotoxin as a specific inhibitor of N-Type calcium channels. An omega conotoxin from *C. magus* venom has been developed into a powerful pain medication called ziconotide, or Prialt (Prommer 2006). It has been seen as a breakthrough because it is 100 to 1000 times more powerful than morphine and lacks morphine‘s addictive properties (Skov et al. 2007). This discovery led to an upsurge in interest regarding the promising future of conotoxin research.

Conus geographus venom has been described as the most potent of all the *Conus* venoms. It has contributed to a number of human fatalities throughout the central Indo-Pacific (Kohn 1958). Contributing to its lethality, but also its therapeutic potential, *C. geographus* venom can inhibit the postsynaptic terminus of the vertebrate neuromuscular junction. Furthermore, the venom has been shown to inhibit muscle action potential propagation even when the muscle is under direct excitation. This inhibition is caused by at least three identified peptides of the *C. geographus* venom: GI, GIA, and GII (Fig 2) (Gray et al. 1981).

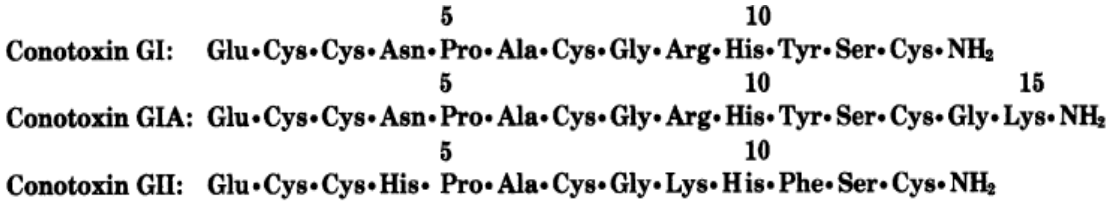

Figure 2: Three monomeric conotoxins that have been tied to the inhibition of the vertebrate neuromuscular junction. Each of these conotoxins contains two internal disulfide bridges, but their location has not yet been identified (Gray et al. 1981).

The wide variety of conotoxins and their vast pharmacological implications have made them a particularly intriguing and useful group of peptides. Furthermore, their combination of specificity and variety suggest that they affect a wide variety of ion channels including T-Type calcium channels. This study was one attempt at finding a conotoxin that can target $Ca_v3.2$ channels. In order to study the effects *C. geographus* venom has on $Ca_v3.2$ channels, RNA containing the $Ca_v3.2$ α1-H pore forming subunit was microinjected into *Xenopus laevis* oocytes. Once expressed in oocytes the $Ca_v3.2$ channels were studied using two-electrode voltage clamp (TEVC) recording techniques. *X. laevis* oocytes that were adequately expressing the $Ca_v3.2$ channels were exposed to *C. geographus* venom and their response to the venom was studied over time. The data presented in this study suggest that *C. geographus* venom contains a conotoxin that partially blocks the $Ca_v3.2$ T-type current. If these results are repeatedly replicated then it is likely that a conotoxin from *C. geographus* venom can have a significant impact on the pharmaceutical treatment of epilepsy, chronic pain, cardiac issues, and other ailments (Perez-Reyes 2006).

Methods

$Ca_v3.2$ Plasmid Isolation

A plasmid containing the $Ca_v3.2$ clone (human heart clone) was provided by Dr. LeAnne Cribbs (Loyola University). The bacteria were cultured for twenty hours in Luria-Bretani broth (1.25g bacto-tryptone, 1.25g NaCl, 0.68g yeast extract, Ampicillin (2μ/ml), and autoclaved water to a volume of 125mL). A Zyppy™ Plasmid Miniprep kit was used to purify the $Ca_v3.2$ DNA. Manufacturer's instructions were followed using 1500 μl of overnight bacterial culture centrifuged at 14000 x g for three minutes. Isolation was tested

using a 1% agarose gel (agarose, 50x TAE buffer, deionized water, SYBR Safe DNA gel stain) and a Promega lambda marker.

$Ca_v3.2$ DNA Linearization and Purification

DNA concentrations were determined using a NanoDrop spectrophotometer. AFL II enzyme (10 units per 1 µg DNA), BSA (Bovine Serum Albumin), NE Buffer, and sterile water were used to linearize the DNA. DNA was heated for four hours at 37˚ C. Linearized DNA was tested with a 1% agarose gel and lambda marker. Linearized DNA was purified using a Biobasic PCR purification kit and manufacturer's instructions. The purified DNA was tested on a 1% Agarose gel to ensure it was the proper size.

$Ca_v3.2$ *In vitro* Transcription and Purification

An Ambion mMessage Machine T7 kit was used according to manufacturer's instructions with 1 µg linear template DNA, Reaction Buffer, NTP/CAP, and RNA polymerase Enzyme Mix. DNA was incubated at 37˚ C for one hour. Transcription was analyzed using 1% agarose gels and concentrations were determined using a NanoDrop spectrophotometer. Transcribed RNA was purified using a Qiagen RNeasy mini kit and tested using a 1% agarose gel. Images for all gels were taken using a Kodak capture program. The high UV setting was used to develop pictures.

Animal Care and Oocyte extraction

Oocytes were extracted from sexually mature female *Xenopus laevis*. *X. laevis* subjects were fed *Xenopus* chow three times per week. Their diet was also supplemented by one goldfish (*Carassius auratus auratus*) per week. Prior to surgery, *X. laevis* subjects were anesthetized with 300 mg MS-222 (3-aminobenzoic acid ester methanesulfate salt) and 200 mL tap water for twenty to forty minutes depending on the size of the subject. They were

euthanized by extended immersion in anesthetic after two surgeries and disposed of per Allegheny College Animal Care guidelines.

Surgical instruments were sterilized with 70 percent ethanol. Oocytes were surgically removed from the *X. laevis* subjects by making an L shaped incision to the right or left of the midline. Removed oocytes were immediately stored in OR-2 solution (82.6 mM NaCl, 2 mM KCl, 5 mM Hepes, 1 mM$MgCl_2$, and water; (pH adjusted to 7.6 with NaOH). After surgery the *X. laevis* muscle was sutured with two sutures and the skin was sutured using three to four sutures.

To separate the oocytes from their connective membrane, they were immersed in 10 mL OR-2 containing collagenase (2mg/mL) at room temperature with shaking for 30 to 60 minutes. They were removed immediately after separation to improve oocyte health and prevent cell membrane decomposition. After being exposed to collagenase, the oocytes were sorted based on optimum appearance (distinct animal and vegetal poles, spherical shape, and relatively large size) and stored in working solution (2.5mL bovine serum/47.5 mL ND-96 (2 mM KCl, 96 mM NaCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM HEPES, pH 7.5) for a maximum of 24 hours at 17˚ C prior to injection.

Oocyte Injection

During microinjection injection needles were used with a diameter of 18-25 μm. Injection needles were pulled using a micropipette puller (Sutter Instruments, model p-97) and were broken by poking an individual pulled piece of injection glass to a Kimwipe stretched over a beaker. Prior to injection, forceps and injection needles were baked at 180˚ C overnight. Two glass slides, microcentrifuge tubes, megapure water, and 10 μL pipette tips were autoclaved for thirty minutes prior to microinjection. The injection needles were back

filled with sterile filtered mineral oil. If the tip was deemed adequate the injection needle was back-filled two thirds of its length. 2μL of sterile water or $Ca_v3.2$ RNA were then drawn into the injection needle using a micro injector. 46 nL of sterile water were injected into each control oocyte and 46 nL $Ca_v3.2$ RNA (.1μg/μl) were injected into each of the experimental oocytes less than 24 hours after harvesting. Oocytes were placed in dishes containing ND-96 during injection and were stored in small Petri dishes (7-15 oocytes per dish) at 17˚ C for five days to allow for $Ca_v3.2$ channel expression. Working solution was changed once per 24 hour period during storage.

Two-Electrode Voltage Clamp (TEVC) Electrophysiology

Glass electrodes were pulled using a micropipette puller (Sutter Instruments, model p-97). Prior to recording, all TEVC wires were cleaned with steel wool and then soaked in bleach until they appeared corroded. Glass electrodes were placed in a beaker containing 3M KCl to fill the tip of the electrode and were then back-filled three quarters the length of the electrode. The resistances for each electrode were tested and were within an accepted range of 0.5-5 MΩ.

TEVC experiments were performed using standard protocol. Standard protocol makes use of one current electrode and one voltage electrode. The voltage electrode measures the voltage across the oocyte membrane and sends a signal to a feedback amplifier. The feedback amplifier then calculates the current needed to achieve a desired membrane potential and sends the required current through the current electrode (Welko 2009).

All TEVC experiments were carried out in a concave recording dish containing 500μl of ND-96 recording solution with an individual oocyte. Prior to recording, the resting membrane potential (RMP) of each oocyte was analyzed. A RMP more hyperpolarized than -

15 mV was considered adequate for recording. A holding RMP equal to the original RMP of the oocyte was set prior to clamp tuning. A 50 mV Seal Test was then used to tune the voltage clamp. During the Seal Test an oscilloscope was used to achieve square waves. After the test the holding potential was adjusted to -60 mV incrementally, so that once placed in clamp at -60 mV, the stress on the oocyte was minimized. To record the current passing through the oocytes, ClampeX 10.1 was used with the Transient-act program. The Transient-act program was used to hyperpolarize the oocyte for 1 secs and then depolarize it in 10mV increments (voltage steps) from -50 to 40 mV for 5 ms. Since TEVC can be very stressful on the oocytes, the RMP of each oocyte was measured immediately after completion of the program to ensure the oocyte survived the procedure. If the electrode resistances remained within the accepted range after the TEVC recording they were reused for the next recording.

***C. geographus* Venom Application and Recording**

If a healthy oocyte (V_m<-20 mV, holding current < 100 nA) showed ideal expression (maximum current < -500 nA) 10 µl (500µg/µl) of crude *Conus geographus* venom was applied to the dish. The venom was reconstituted by adding 10µl ND-96 recording solution to the venom immediately before use. Then the Transient-act program was run at zero, five, ten, and fifteen minutes. At the completion of the program the electrodes and ND-96 containing the venom were swapped with fresh counterparts for future recordings of different oocytes.

Water-Subtraction and Normalization

To control for naturally occurring currents in the oocytes, the average current of the water-injected oocytes (N=3) at each voltage step was subtracted from coinciding currents of the $Ca_v3.2$ RNA injected oocytes. After water subtraction each $Ca_v3.2$ RNA injected oocyte

was normalized to accurately compare different oocytes. Normalization was performed by dividing the current at each voltage step post-venom application by the maximum current observed at any voltage step in the same oocyte prior to venom application. The same normalization procedure was performed for pre-venom application as well.

Data Analysis

Data was analyzed using paired two sample one tail T-Tests with:

H_o: Mean $_{\text{Maximum Current No Venom}} \leq$ Mean $_{\text{Maximum Current With Venom}}$

H_A: Mean$_{\text{Maximum Current No Venom}} >$ Mean$_{\text{Maximum Current With Venom}}$

A p-value less than 0.05 was considered significant.

NOTE: After defense it was determined that ANOVA statistical analysis should be performed for future analysis across voltage steps.

Results

Molecular Preparation and Analysis

Two $Ca_v3.2$ DNA samples were purified from linearized DNA. Purified samples were analyzed using a 1% Agarose gel (Fig 3). The presence of a band between 9.4 and 23.1 kbps was observed and indicated successful purification of $Ca_v3.2$ DNA (known molecular weight 10.1 kbps). The concentrations of the two samples were determined to be 181.9 ng/µl and 117.3 ng/µl by spectrophotometric analysis.

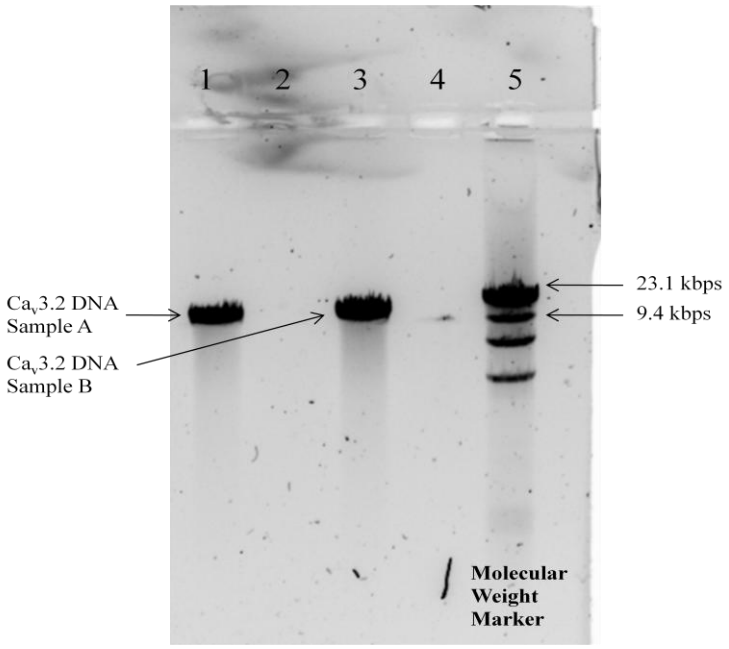

Figure 3: 1% Agarose gel indicating successful DNA purification of linearized Cav3.2 DNA. Lane 1 contains purified $Ca_v3.2$ DNA Sample A (181.9 ng/µl). Lane 3 contains purified $Ca_v3.2$ DNA Sample B (117.3 ng/µl). Lane 5 contains the lambda DNA molecular weight marker (Promega) with key bands indicated.

$Ca_v3.2$ RNA was transcribed and purified from purified DNA (181.9 ng/µl). The concentration of the transcribed $Ca_v3.2$ RNA was 4126.2 ng/µl. After purification the sample was analyzed using a 1% Agarose gel (Fig 4). The resulting band between 7 and 5 kbps was expected and indicated successful purification and transcription. Spectrophotometric analysis indicated a purified RNA concentration of 102.6 ng/µl.

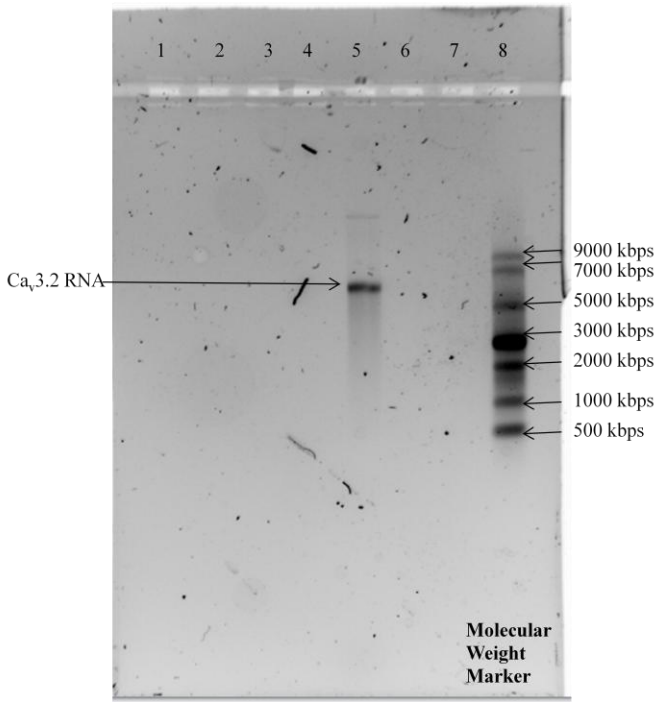

Figure 4: 1% Agarose gel indicating successful purification of Cav3.2 RNA. Lane 5 contains purified Cav3.2 RNA (102.6 ng/µl). Lane 8 contains the RNA molecular weight marker (New England Bio Labs) with key bands indicated.

Oocyte Survival and Expression

Fifteen water-injected oocytes were analyzed and four were found to have survived injection (26.67% survival rate). Three of the four surviving water injected oocytes were healthy enough to be exposed to venom. 94 Ca$_v$3.2 RNA injected oocytes were analyzed and 23 survived injection (24.47% survival rate). When put into clamp, fifteen Ca$_v$3.2 RNA injected oocytes showed strong expression of the channel. Of those fifteen, eight met the criteria for venom application (Table 1).

Table 1: Summary table of all oocytes analyzed in this study. Oocytes were considered healthy if their resting membrane potential was more hyperpolarized than -15 mV. Healthy oocytes that were considered to be showing strong channel expression had a maximum inward current of less than -500 nA. Venom application criteria were outlined in the methods section.

Type Of Oocyte	**Water-Injected**	**$Ca_v3.2$ RNA-Injected**
Total Analyzed	15	94
Total Healthy (V_m<-15mV)	4	23
Total Meeting Venom Application Criteria	3	8
Total Showing Strong Channel Expression (Maximum Current < -500 nA)	N/A	15

All $Ca_v3.2$ RNA injected oocytes were considered expressing properly if the current matched the characteristic shape for $Ca_v3.2$ channels. The shape is indicated by a negative dip that indicates inward flow of calcium ions (Fig 5). Maximum inward current was observed at either -50 or -40 mV during electrophysiological recordings of all analyzed oocytes.

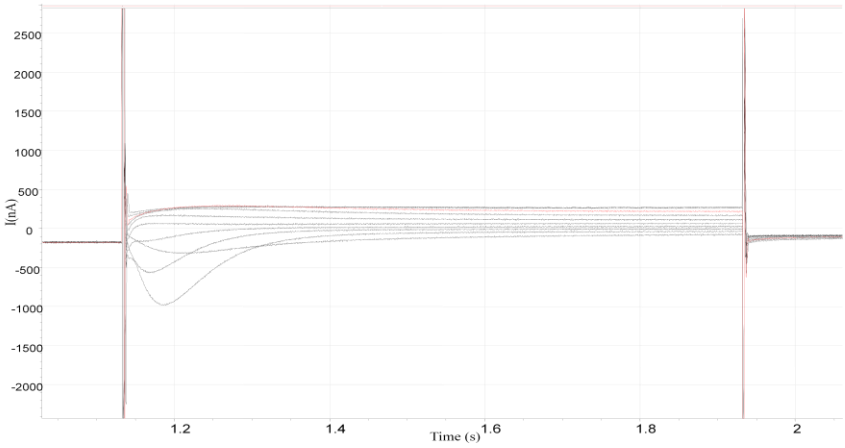

Figure 5: Current trace of an oocyte expressing $Ca_v3.2$ channels. Maximum current is found at the bottom of the most negative dip. The maximum current of this oocyte was observed at -982 nA during the -40 mV voltage step. The X-Axis represents the time in seconds and the Y-Axis represents the current in nA. Each line corresponds to a voltage step as described in the methods section.

The characteristic $Ca_v3.2$ shape is not observed in water-injected oocytes. Ideal water-injected oocytes do not show peaks or dips and are indicated by a group of non-crossing lines with a positive maximum current, representing a small outward flow of ions. (Fig 6).

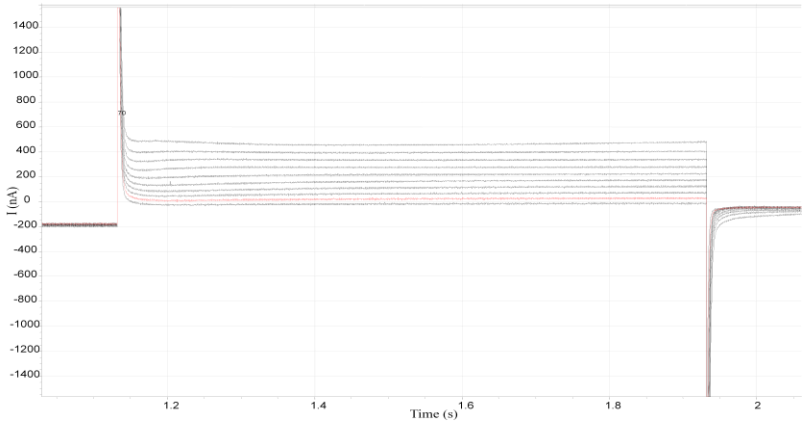

Figure 6: Current trace of a water injected oocyte. Maximum current is found at the most positive line indicating outward flow of ions. The maximum current of this oocyte was observed at 509 nA during the +40 mV voltage step. The X-Axis represents the time in seconds and the Y-Axis represents the current in nA. Each line corresponds to a voltage step as described in the methods section.

Electrophysiological Recordings

Normalization was performed on all $Ca_v3.2$ RNA injected oocytes after water-subtraction to account for differences between individual oocytes. All $Ca_v3.2$ RNA injected oocytes that were normalized and exposed to *C. geographus* venom were averaged and compared to pre-exposure recordings over time. At all voltages, and at all time trials, the normalized data showed either no effect to or a decrease in inward current (Fig 7).

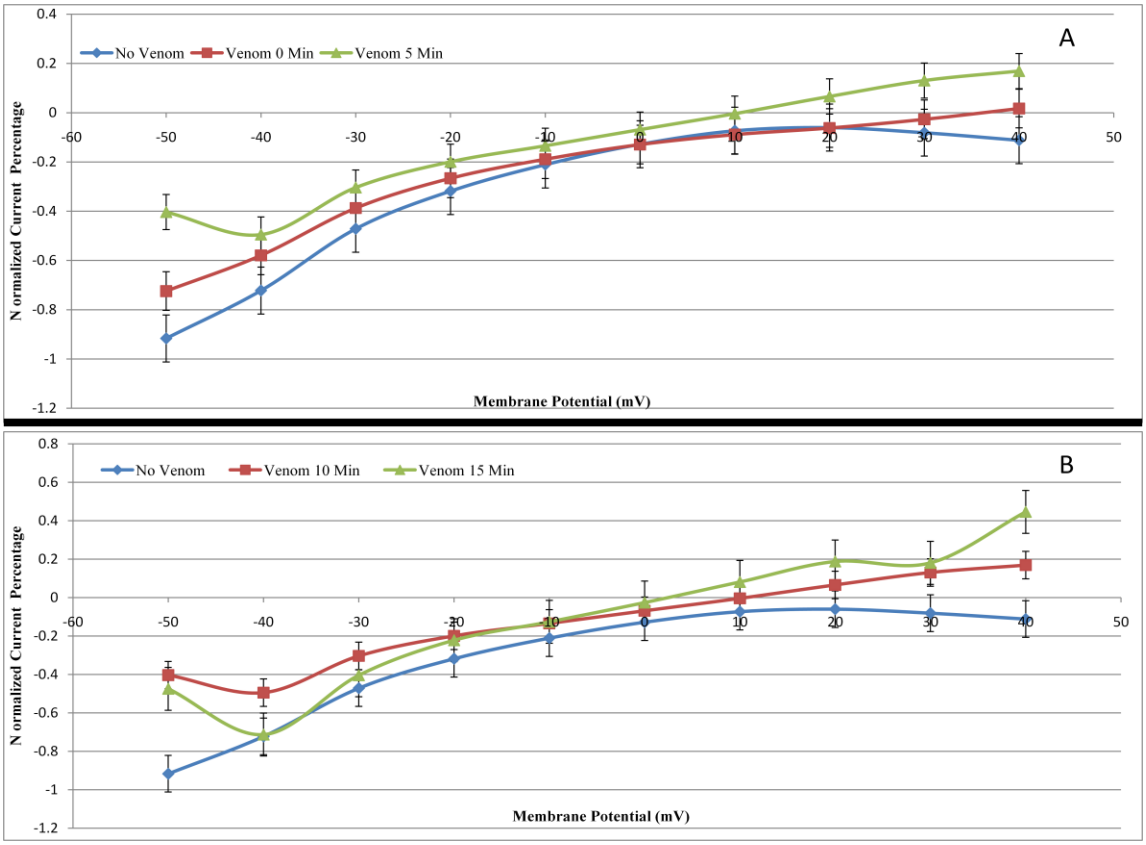

Figure 7: Normalized current vs. membrane potential (mV) in the presence of *C. geographus* venom. Panel A shows the normalized current at each voltage step in an unexposed oocyte (Blue), initially after venom exposure (Red), and five minutes after venom exposure (Green). Panel B shows the normalized current percentage at each voltage step in an unexposed oocyte (Blue), ten minutes after venom exposure (Red), and fifteen minutes after venom exposure (Green). In both panels the X-Axis represents the membrane potential at voltage steps from -50 mV to 40 mV. The Y-Axis is the normalized current percentage. A negative value indicates inward ion flow and a positive value indicates outward ion flow. Error bars represent standard error of the mean.

Since all analyzed oocytes had a maximum inward current at either -40 mV (N=1) or -50 mV (N=7) these two membrane potentials were used for data analysis. Of all the oocytes that were exposed to venom, some were not of optimum health (N=2) and were discarded from statistical analysis. They were discarded due to either a lower than normal resting membrane potential (V_m<-40 mV) or a significantly higher than average holding current (greater than 100 nA) during the clamping procedure. Prior to venom application, the average normalized current at -50 mV and -40 mV were -0.889 and -0.697 respectively (N=6). At -50 mV the average inward current decreased by 17.8 percent immediately after venom exposure, 68.3 percent five minutes post exposure, 71.9 percent ten minutes post exposure, and 60.7 percent fifteen minutes post exposure. At -40 mV the average inward decreased by 24.5 percent immediately after venom exposure, 50.3 percent five minutes post exposure, 40.1 percent ten minutes post exposure, and 23.3 percent fifteen minutes post exposure (Fig 8).

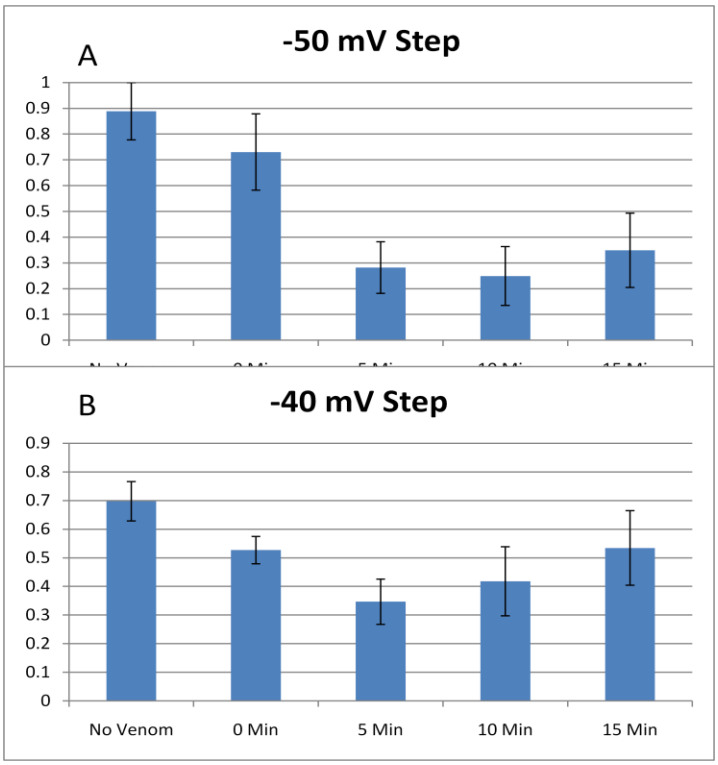

Figure 8: Normalized current of oocytes prior to *C. geographus* venom exposure, immediately after venom exposure, five minutes post venom exposure, ten minutes post venom exposure, and fifteen minutes post venom exposure. The X-Axis indicates the venom exposure time and the Y-Axis is the normalized current percentage. Panel A consists of the average data for oocytes during the -50 mV voltage step. Panel B consists of the average data for oocytes during the -40 mV voltage step. Error bars represent standard error of the mean.

To determine if these results were statistically significant, one-tailed paired T-Tests were performed on the six selected oocytes. At -50 mV the results were found to be statistically significant at five ($p=0.0005$), ten ($p=0.0004$), and fifteen ($p=0.003$) minutes post venom exposure. The results were not statistically significant initially after venom exposure ($p>0.05$) ($N=6$). At -40 mV the results were found to be statistically significant at five minutes post venom exposure ($p=0.01$), but not immediately after venom exposure, ten minutes post venom exposure, or fifteen minutes post venom exposure ($p>0.05$, $N=6$). Furthermore, the effects of the venom were not significant in water injected oocytes at -50

and -40 mV ($p>0.05$, $N=3$).

The calcium channels were completely blocked by *C. geographus* venom in one particular oocyte. This oocyte was one of the healthiest oocytes observed (V_m=-50mV, $I_{Holding}$= -25.9 nA) and remained healthy throughout recording (Final V_m= -35 mV). The maximum current at -40 mV prior to venom exposure, zero, five, ten, and fifteen minutes post exposure was -1104.73 nA, -921.63 nA, -134.28 nA, -18.31 nA, and 15.26 nA respectively (Fig 9). Similar effects were observed during the -50 mV step, but the picture was omitted.

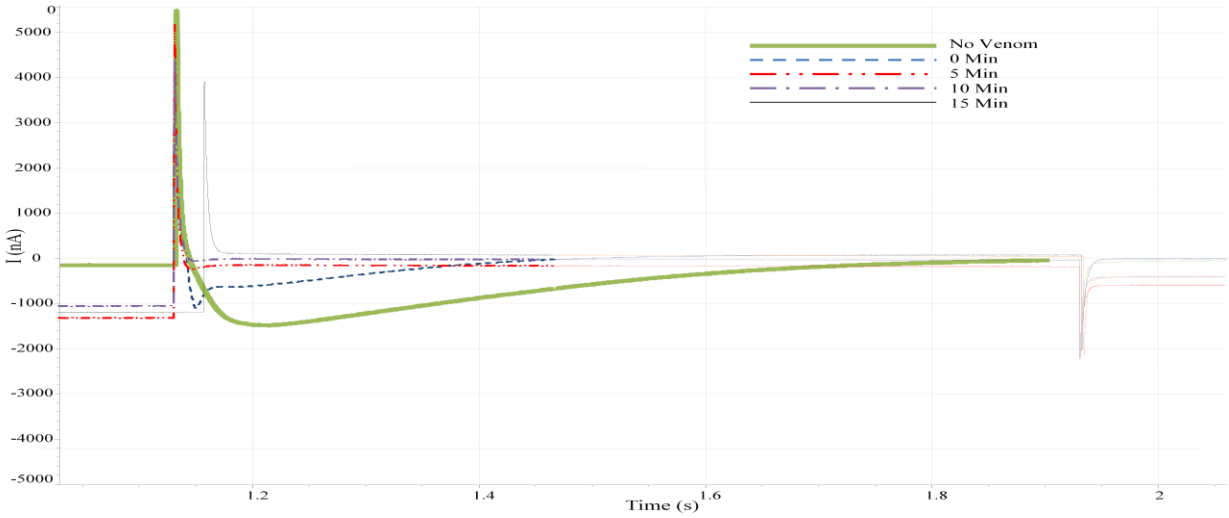

Figure 9: Current trace of an oocyte in response to *C. geographus* venom pre-exposure and zero, fifteen, ten, and fifteen minutes post-exposure. The X-Axis represents the time in seconds and the Y-Axis represents the current in nA. Each line corresponds to a voltage step as described in the methods section.

Discussion

In this study I analyzed the effects of *C. geographus* venom on $Ca_v3.2$ channels over time using two-electrode voltage clamp (TEVC) techniques. Purified $Ca_v3.2$ RNA was injected into *Xenopus laevis* oocytes and given five days to express. After expression was confirmed, *C. geographus* venom was applied and a program was run using TEVC that depolarized the oocyte from -50 to 40 mV in 10 mV increments.

Previous studies in the French lab that utilized $Ca_v3.2$ channels and *X. laevis* oocytes encountered problems regarding the maintenance of healthy oocytes. During this study, issues with oocyte health were resolved through reformulation of the oocyte care technique. The first modification was to the amount of time oocytes were exposed to collagenase after their removal from *X. laevis* subjects. In previous studies oocytes were exposed to collagenase for a total of one hour (Welko 2009). During this study it was determined that an exposure time limited to thirty minutes improved oocyte health and resulted in more frequent expression of the $Ca_v3.2$ channels after five days. Another method that enhanced oocyte survival and expression likelihood was the modification of the amount of oocytes stored in each small Petri dish during incubation. Improved survival and expression rates were observed when 7-10 oocytes occupied each dish, as opposed to the 10-20 used previously (Welko 2009). The resolution of oocyte health issues proved extremely beneficial towards obtaining usable data. Prior to modifying the protocol similar difficulties to past studies were observed (data not shown).

After exposing the $Ca_v3.2$ RNA injected oocytes to *C. geographus* venom, a decrease in inward current was observed at both -40 and -50 mV (Fig 8). This decrease was observed

at all exposure times at both -40 and -50 mV, but varied in statistical significance. The results were statistically significant at zero, five, and fifteen minutes post venom exposure at -50 mV, but were only significant five minutes post exposure during at -40 mV. $Ca_v3.2$ inhibition at both -40 and -50 mV was statistically insignificant immediately after venom exposure.

The data suggest that *C. geographus* venom inhibits $Ca_v3.2$ channel function at both -40 and -50 mV. The complete reversal of inward current over time in one $Ca_v3.2$ RNA injected oocyte further supports this claim (Fig 9). The results of past senior projects in the French lab have found conflicting evidence. Welko reported that *C. geographus* venom had an insignificant effect on $Ca_v3.2$ channels expressed in *Xenopus* oocytes (2009). Her findings are relevant to this study, but her issues with oocyte health and small sample size (N=2) make her data somewhat unreliable. My study also used more recently obtained venom samples (obtained in 2010) than the Welko report (venom obtained in 2002 and reconstituted in two batches-2002 and 2007). It was possible that Welko was utilizing venom samples that degraded over time.

The inhibition of the $Ca_v3.2$ channels by *C. geographus* venom at -40 and -50 mV is particularly important because the channels are naturally activated at -46 mV (Klöckner et al. 1999). *C. geographus* venom continues to block $Ca_v3.2$ channels for fifteen minutes at a membrane potential of -50 mV, but does not have a statistically significant effect on the channels after ten and fifteen minutes at -40 mV. This may be attributed to a few different factors.

As it has been mentioned, the channels are naturally active at -46 mV. It is possible that the venom targets the channels at more hyperpolarized membrane potentials by affecting

the S4 transmembrane segment of the α-1 subunit (Dirkson et al. 1997).. Since the S4 segment acts as the voltage sensor it is possible that the venom modifies the sensor to reduce inward current at membrane potentials of -50 mV, but does not have as pronounced of an effect on the inward current at the -40 mV step.

C. geographus venom has been previously shown to have varied blocking capabilities. Feldman et al. (1987) reported that the blocking capabilities of one omega *C. geographus* conotoxin, GVIA, improved as the cell membrane was depolarized. It is possible that the crude venom focuses on membrane potentials closer to -50 mV where the transient signals begin to activate (Carbone 1984). By focusing on the -50 mV step the *C. geographus* snails can stop the transport of calcium ions at lower membrane potentials, which is ultimately inhibiting the ability of T-Type channels to create initial signaling currents. The hunting mechanism of *C. geographus* may have also been a factor the decrease of observable inhibition at -40 mV, but not -50 mV. *C. geographus* is a piscivorous snail that rapidly paralyzes and kills its prey (Terlau and Olivera 2004). It is unlikely that *C. geographus* would need to develop venom that has long-lasting effects because it requires mere seconds, not minutes, to paralyze its victim.

One possible way that the effects of the venom can appear to diminish over time is through the recovery of the oocyte. Throughout this assay oocyte health improved over time after the initial insertion of recording electrodes into the membrane of the oocyte (data not shown). It is possible that healing over time led to an increase in current due to the improvement of the health of the oocyte. Healing would cause the appearance of venom having diminishing effects, but would not be related to the venom considering water injected oocytes experienced no significant current or health effects in response to the venom.

Ultimately it seems that this difference is a result of insufficient sample size. Previous studies have suggested the effects of individual *C. geographus* conotoxins do not decline over short periods of time (<30 min) (Kerr and Yoshikami 1984; Feldman et al. 1987). Insufficient sample size is a likely cause of statistically insignificant data considering the inhibition of the channel was empirically observed at each time period at both -50 mV and -40 mV (Fig 8). A larger sample size could improve statistical results, and is one suggestion for future studies regarding the effect of *C. geographus* venom on $Ca_v3.2$ channels over time.

The inhibition of inward current experienced over time may have been due to a variety of potential mechanisms affecting the α-1 subunit. The first of which is the use of redox reactions by the venom. Redox reactions transfer electrons from one compound to another changing the oxidation state of a molecule and thus changing its functionality. Nelson et al. found that ascorbate selectively blocked $Ca_v3.2$ channels exclusively. They suggested that metal catalyzed oxidation was the likely cause of inhibition by ascorbate. This was determined by the presence of 2-oxo-histidine, which is a result of the oxidation of a unique, metal-binding, histidine residue in domain 1 of $Ca_v3.2$ channels (Nelson et al. 2007). Other studies have also suggested redox reactions as $Ca_v3.2$ channel inhibitors (Lee et al. 2009). Todorovic et al. found that 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNP) and mibefradil inhibited $Ca_v3.2$ channels involved in peripheral nociception of rats through redox reactions (2001). Since many *C. geographus* conotoxins are charged compounds it is possible that they utilize redox reactions as an agent to inhibit T-Type calcium channel function (Lew et al. 1997; Cruz et al. 1984).

Another possibility for the inhibition of T-Type channel function by *C. geographus* venom is the use of binding sites that modify the shape of the pore in the α-1 subunit. This is

suggested as the cause for the popular nickel block that $Ca_v3.2$ channels are notoriously sensitive to. Kang et al. found evidence to support the hypothesis that nickel binds to the S4-S5 linkage of the α-1 subunit. During their study when nickel was bound it effectively prevented the gating of $Ca_v3.2$ channels and left them in a perpetually closed state (Kang et al. 2006). In N-Type channels, some conotoxins have been shown to bind directly to a binding site at the entrance of the pore. Through this binding they physically obstruct the passage of ions through the pore (Liang and Elmslie 2002; Ellinor et al. 1994). These types of inhibitory mechanisms are particularly useful for pharmacological reasons because they are concentration dependent. Perez-Reyes et al. (2009) found that dihydropyridine inhibited $Ca_v3.2$ channel function by binding to a specific binding site on the α-1 subunit, but that this effect was dependent on concentration.

This assay suggests the possibility that a specific inhibitor of $Ca_v3.2$ channels can be found in *C. geographus* venom. If that possibility remains after repeated study, then it is possible that a therapeutic drug for epilepsy, cardiac hypertrophy, cancer cell proliferation, and neuropathic pain may be developed from a conotoxin within *C. geographus* venom (Nielsen et al. 2000). To properly identify $Ca_v3.2$ channel inhibitors within *C. geographus* venom, future studies must be performed with fractions of the venom. The fractionation of the venom by chromatography will be necessary to identify specific conotoxins within the venom that can target $Ca_v3.2$channels.

Works Cited

Bear M., Connors B., and Paradiso M., Neuroscience: Exploring the Brain. 2nd ed. N.p.: Lippincott Williams & Wilkins, 2002.

Carbone, E., and H. D. Lux. "A Low Voltage-Activated, Fully Inactivating Ca Channel in Vertebrate Sensory Neurones." *Nature* 310.5977 (1984): 501-2. Print.

Catterall, W. A. "Structure and Regulation of Voltage-Gated Ca2+ Channels." *Annual Review of Cell and Developmental Biology* 16 (2000): 521-55. Print.

Catterall, W. A., et al. "International Union of Pharmacology. XLVIII. Nomenclature and Structure-Function Relationships of Voltage-Gated Calcium Channels." *Pharmacological reviews* 57.4 (2005): 411-25. Print.

Cruz, L. J., et al. "Conus Geographus Toxins that Discriminate between Neuronal and Muscle Sodium Channels." *The Journal of biological chemistry* 260.16 (1985): 9280-8. Print.

Dirksen, R. T., et al. "The S5-S6 Linker of Repeat I is a Critical Determinant of L-Type Ca2+ Channel Conductance." *Biophysical journal* 73.3 (1997): 1402-9. Print.

Dolphin, A C. "A short history of voltage-gated calcium channels." British Journal of Pharmacology 147.S1 (2006): S56-62.

Ellinor, P. T., et al. "Structural Determinants of the Blockade of N-Type Calcium Channels by a Peptide Neurotoxin." *Nature* 372.6503 (1994): 272-5. Print.

Fatt, P, and B Katz. "The electrical properties of crustacean muscle fibres." J Physiol 120.1-2 (1953): 171-204.

Feldman, D. H., B. M. Olivera, and D. Yoshikami. "Omega Conus Geographus Toxin: A Peptide that Blocks Calcium Channels." *FEBS letters* 214.2 (1987): 295-300. Print.

Gray, W. R., et al. "Peptide Toxins from Conus Geographus Venom." *The Journal of biological chemistry* 256.10 (1981): 4734-40. Print.

Hammond, Constance. *Cellular and Molecular Neurophysiology*. 3rd ed. N.p.: Academic Press, 2008. Print.

Hildebrand, M. E., et al. "A Novel Slow-Inactivation-Specific Ion Channel Modulator Attenuates Neuropathic Pain." *Pain* 152.4 (2011): 833-43. Print.

Hille, Bertil. Ion Channels of Excitable Membranes. N.p.: University of Washington, 2001.

Hofmann, F., M. Biel, and V. Flockerzi. "Molecular Basis for Ca2+ Channel Diversity." *Annual Review of Neuroscience* 17 (1994): 399-418. Print.

Hofmann, F., L. Lacinova, and N. Klugbauer. "Voltage-Dependent Calcium Channels: From Structure to Function." *Reviews of physiology, biochemistry and pharmacology* 139 (1999): 33-87. Print.

Huxley, A. F. "Hodgkin and the Action Potential 1935-1952." *The Journal of physiology* 538.Pt 1 (2002): 2. Print.

Jin-Ah Kim, Jin-Yong Park, Ho-Won Kang, Sung-Un Huh, Seong-Woo Jeong, and Jung-Ha Lee. "Augmentation of Cav3.2 T-Type Calcium Channel Activity by cAMP-Dependent Protein Kinase A." The Journal of Pharmacology and Experimental Therapeutics. 318.1 (2006)

Kang, H. W., et al. "A Molecular Determinant of Nickel Inhibition in Cav3.2 T-Type Calcium Channels." *The Journal of biological chemistry* 281.8 (2006): 4823-30. Print.

Kerr, L. M., and D. Yoshikami. "A Venom Peptide with a Novel Presynaptic Blocking Action." *Nature* 308.5956 (1984): 282-4. Print.

Klockner, U., et al. "Comparison of the Ca2 + Currents Induced by Expression of Three Cloned alpha1 Subunits, alpha1G, alpha1H and alpha1I, of Low-Voltage-Activated T-Type Ca2 + Channels." *The European journal of neuroscience* 11.12 (1999): 4171-8. Print.

Kohn, A. J. "Cone Shell Stings; Recent Cases of Human Injury due to Venomous Marine Snails of the Genus Conus." *Hawaii medical journal* 17.6 (1958): 528-32. Print.

Lee, W. Y., et al. "Molecular Mechanisms of Lipoic Acid Modulation of T-Type Calcium Channels in Pain Pathway." *The Journal of neuroscience : the official journal of the Society for Neuroscience* 29.30 (2009): 9500-9. Print.

Lew, M. J., et al. "Structure-Function Relationships of Omega-Conotoxin GVIA. Synthesis, Structure, Calcium Channel Binding, and Functional Assay of Alanine-Substituted Analogues." *The Journal of biological chemistry* 272.18 (1997): 12014-23. Print.

Liang, H., and K. S. Elmslie. "Rapid and Reversible Block of N-Type Calcium Channels (CaV 2.2) by Omega-Conotoxin GVIA in the Absence of Divalent Cations." *The Journal of neuroscience : the official journal of the Society for Neuroscience* 22.20 (2002): 8884-90. Print.

Luo, S., Zhangsun, D., Wu, Y., Zhu, X., Xie, L., Hu, W., Zhang, J. and Zhao, X.. Identification and Molecular Diversity of T - superfamily Conotoxins from Conus lividus and Conus litteratus." Chem Biol Drug Des 2006; 68: 97–106.

Monteil, A., et al. "Molecular and Functional Properties of the Human Alpha(1G) Subunit that Forms T-Type Calcium Channels." *The Journal of biological chemistry* 275.9 (2000): 6090-100. Print.

Nelson, M. T., et al. "Molecular Mechanisms of Subtype-Specific Inhibition of Neuronal T-Type Calcium Channels by Ascorbate." *The Journal of neuroscience : the official journal of the Society for Neuroscience* 27.46 (2007): 12577-83. Print.

Nichols, D. *Proteins, Transmitters, and Synapses.* Oxford, England: Blackwell Scientific Publications, 1994. Print.

Nielsen, K J., T Schroeder, and R Lewis. "Structure-activity relationships of omega-conotoxins at N-type voltage-sensitive calcium channels." Mol. Recognit 13.2 (2000): 55-70.

Olivera, B M. "E.E. Just Lecture, 1996. Conus venom peptides, receptor and ion channel targets, and drug design: 50 million years of neuropharmacology." Mol Biol Cell 8.11 (1997): 2101-09.

Perez-Reyes, E. "Molecular Characterization of T-Type Calcium Channels." *Cell calcium* 40.2 (2006): 89-96. Print.

Perez-Reyes, E., et al. "Molecular Characterization of a Neuronal Low-Voltage-Activated T-Type Calcium Channel." *Nature* 391.6670 (1998): 896-900. Print.

Perez-Reyes, E., A. L. Van Deusen, and I. Vitko. "Molecular Pharmacology of Human Cav3.2 T-Type Ca2+ Channels: Block by Antihypertensives, Antiarrhythmics, and their Analogs." *The Journal of pharmacology and experimental therapeutics* 328.2 (2009): 621-7. Print.

Prommer, E. "Ziconotide: a new option for refractory pain." Drugs Today 42.6 (2006): 369-78.

Purves, Dale, George J. Augustine, David Fitzpatrick, William C. Hall, and Anthony-Samuel LaMantia. Neuroscience. 4th ed. N.p.: Sinauer Associates, 2007. 156-57

Raybaud, A., et al. "The Role of Distal S6 Hydrophobic Residues in the Voltage-Dependent Gating of CaV2.3 Channels." *The Journal of biological chemistry* 282.38 (2007): 27944-52. Print.

Skov, M J., J C. Beck, A W. de Kater, and G M. Shopp. "Nonclinical safety of ziconotide: an intrathecal analgesic of a new pharmaceutical class"." Int. J. Toxicol. 26.5 (2007): 411-21

Snutch, T. P., and S. M. Cain. "Contributions of T-Type Calcium Channel Isoforms to Neuronal Firing." *Channels (Austin, Tex.)* 4.6 (2010): 475-82. Print.

Spedding, M., and R. Paoletti. "Classification of Calcium Channels and Calcium Antagonists: Progress Report." *Cardiovascular drugs and therapy / sponsored by the International Society of Cardiovascular Pharmacotherapy* 6.1 (1992): 35-9. Print.

Todorovic, S. M., et al. "Redox Modulation of T-Type Calcium Channels in Rat Peripheral Nociceptors." *Neuron* 31.1 (2001): 75-85. Print.

Terlau, H., and B. M. Olivera. "Conus Venoms: A Rich Source of Novel Ion Channel-Targeted Peptides." *Physiological Reviews* 84.1 (2004): 41-68. Print.

Vassort, G, and J Alvarez. "Cardiac T-type calcium current: pharmacology and roles in cardiac tissues."J Cardiovasc Electrophysio 5.4 (1994): 376- 93.

Welko, A. "The effects of specific Conus snail venoms on the T-type calcium channel Cav3.2." Allegheny College (2009).

Yunker, A. and McEnery M. (2003). Low-voltage-activated (T-Type) calcium channels in review. Journal of Bioenergetics and Biomembranes 33(6): 533-575.