1-1-2010

The M-Superfamily of Conotoxins: A Review

Reed B. Jacob
Boise State University

Owen M. McDougal
Boise State University

The M-superfamily of Conotoxins: A Review

Reed B. Jacob and Owen M. McDougal
Boise State University

Abstract
The focus of this review is the M-superfamily of *Conus* venom peptides. Disulfide rich peptides belonging to the M-superfamily have three loop regions and the cysteine arrangement: CC-C-C-CC, where the dashes represent loops one, two, and three, respectively. Characterization of M-superfamily peptides has demonstrated that diversity in cystine connectivity occurs between different branches of peptides even though the cysteine pattern remains consistent. This superfamily is subdivided into five branches, M-1 through M-5, based on the number of residues in the third loop region, between the fourth and fifth cysteine residues. M-superfamily peptides appear to be ubiquitous in *Conus* venom. They are largely unexplained in indigenous biological function and they represent an active area of research within the scientific community.

Keywords
M-superfamily, conotoxin, review, marine snail, venom, peptide.

Introduction

Throughout the world there exist both predator and prey. This distinction is apparent though sometimes misleading. Take for example marine snails of the genus *Conus* that are present across the oceans of the southern hemisphere [1]. These snails are slow moving animals that appear more prey than predator. However, they have evolved into effective predators through the development of venom consisting of biologically active peptides. The venom is loaded into a hollow harpoon that the snail injects into the intended prey: fish, worms, or other snails [2]. The categories of cone snails based on prey preference are piscivorous (fish eating), molluscivorous (mollusk eating), and vermivorous (worm eating) [3]. The cone snail venom contains myriad peptide components significant to the survival of the organism with respect to hunting and defense against being eaten [4]. Interest by researchers in snails of the genus *Conus* began in the early nineteen seventies as evidence of their involvement in numerous human fatalities mounted [5]. Dr. Alan Kohn, an early pioneer in the study of hunter/prey relationship of cone snails, recognized that the venom of cone snails may possess therapeutic components [6]. During that time, Dr. Robert Endean and coworkers in Australia demonstrated that the venom of dissimilar species of cone snail contained a diversity of biologically active components. Dr. Baldomero (Toto) Olivera and coworkers at the University of Utah became the primary innovators of successful laboratory techniques in the study of venom components extracted from cone snails [7]. Foremost among these innovations was an avant-garde method of bio-assay using intracranial rather than intraperitoneal injection of toxin into subject mice. This new delivery method revealed greater sensitivity to individual peptides in fish and mouse studies than those from standard M-superfamily intraperitoneal injections [8]. This early research revealed the disulfide rich nature of the majority of peptide components from *Conus* snail venom. The disulfide rich peptides became broadly defined as conotoxins [9].

From Conotoxins to Drugs. The intracranial injection method of conotoxin delivery into fish and mouse subjects allowed researchers to begin unraveling the complicated chemistry of the neurotoxic peptides and paving the path for therapeutic applications. The venom of any individual cone snail contains upwards of 100 different peptides; each with a distinctive role when injected into the target subject. It is the cumulative effect of the individual peptides that causes the venom to be deadly to the prey. Researchers called the collective effect of the venom a “cabal”, after unspecified covert groups organized to overthrow equally unspecified governments [10]. With different snails, researchers noted different cabals: the ‘lightning-strike cabal’, the ‘motor cabal’, and the ‘nirvana cabal’, named after the general set of reactions elicited by the overall effect of the venom on the test subject. The ‘lightning-strike cabal’ inhibits muscular contraction in prey due to a combination of paralytic peptides that act to block voltage-gated Na^+ and K^+ channels; this has an effect similar to electrocution. The ‘motor cabal’ effectively inhibits the pre-synaptic Ca^{2+} channels, post-synaptic nicotinic receptors and Na^+ channels. The combination achieves total

inhibition of neuromuscular transmission [11]. The 'lightning-strike' and 'motor' cabals often work in conjunction. The 'nirvana cabal' diminishes the sensory circuitry of the prey by producing a euphoric effect [5]. This allows the snail to capture its prey in a net-like extendable stomach for consumption. Active research in the field of conotoxins involves the isolation, identification, and assessment of biological activity for individual peptides that possess the potential to be among the most potent and selective therapeutics ever studied.

Conotoxin components in the venom target myriad receptor sites in the prey, a predatory tactic that has been adopted across mammalia, from "*Caenorhabditis elegans* to humans" [12]. Individual conotoxins range between 10-100 amino acids in length [2]. The genus *Conus* contains over 700 species, representing a peptide library on the order of 70,000 sequences. Conotoxins represent extremely specific biological probes that offer researchers a tool to understand and differentiate between closely related receptors [13]. The simplicity of conotoxins has made them valuable in the advancement of neuroscience research and consequent drug development [14]. Conotoxins are difficult to isolate from the venom, but once sequenced and partially reduced, scientists have been able to synthesize and properly fold (disulfide bonds) synthetic peptides for their investigations. Currently, only about 0.2% of the conotoxin peptide library has been cataloged [9, 15].

Many diseases, such as epilepsy, Schizophrenia, Tourette's Syndrome, Parkinson's disease and sclerosis, are associated with improper functioning of signal channels. Conotoxin-based therapeutics have demonstrated great promise because they are relatively small, potent, selective antagonists and agonists of specific cell membrane channel proteins [16]. A wide range of companies, in Australia, Ireland, and the United States, are currently developing and testing drugs based on conotoxins and/or conotoxin molecular scaffolds. Examples of conotoxin derived drugs either in clinical trials or commercially available include ACV1 [17], AM336 [18], Prialt™ [15, 19-23], CGX-1160, CGX-1007, and CGX-100 [24]. At this time the only conotoxin drug approved by the United States Food and Drug Administration for public use is Prialt™. Prialt™ is a synthetic conopeptide derived from *Conus magus* used to treat chronic pain and is one of the most powerful pain therapeutics known to date [15]. Prialt™ is the trade name for ω-conotoxin MVIIA, an N-type calcium channel blocker. This drug provides a non-addictive means to block pain in subject patients by inhibiting the source of pain transmission in nerve cells of the spinal cord. For this reason it is a desirable alternative to traditional opiate derivatives like morphine or codeine [25].

An Introduction into the M-superfamily of Conotoxins: Members of the M-superfamily of conotoxins have been found in every *Conus* species tested to date [4]. Classification of this widely divergent superfamily is based on the general pattern derived from the number of residues contained in each of the three loop regions $CC(X_{4-6})C(X_{4-5})C(X_{1-5})CC$, where X_{4-6} represents four to six amino acids in the first loop, X_{4-5} represents four to five amino acids in the second loop, and X_{1-5} represents one to five amino acids in the third loop [26]. M-superfamily peptides are further divided into 5 branches, labeled M-1 to M-5, based on the number of residues that exist in the third cystine loop between the fourth and fifth cysteine residues. A further delineation separates the five branches into the Mini-M and Maxi-M conotoxins, where M-1 through M-3 are considered Mini-M's and M-4/M-5 are Maxi-M's (Figure 1). This differentiation is based on the overall number of residues in the mature peptide. The Mini-M conotoxins all contain fewer than 22 residues, while the Maxi-M peptides contain more than 22 amino acids [13]. The Maxi-M (M-4 and M-5) peptides are sub-grouped into μ-, ψ-, and κM-conotoxins based on their biological targets. The μ-conotoxins block voltage-gated sodium channels, κM-conotoxins block voltage-gated potassium channels in muscle [2], and ψ-conotoxins block nicotinic acetylcholine receptors [26]. Members of the M-superfamily for which a target receptor is known are exclusively within the M-4 and M-5 branches with relatively little known about the biological receptors targeted by the Mini-M peptides. It is known that the target receptor for the Mini-M peptides is different from that of the Maxi-M peptides [26].

-Figure 1-

Mini-M's: Interestingly, the Mini-M peptides demonstrate unique disulfide bond connectivity patterns, loop size variability, and unique 3D folding structure even within the same branch. The M-superfamily is the only superfamily where such diverse structure and cysteine connectivity is present, especially in the Mini-M's, M-1 through M-3.

M-1 branch: The lack of solid mechanistic evidence of activity and definitive target receptor has hampered the comprehensive publication of the Mini-M peptides. Much of what is presently known of the M-1 branch has been through the study of conotoxins mr3e (from *C. marmoreus*) and tx3a (from *C. textile*). Conotoxin mr3e has the disulfide connectivity Cys1-Cys5, Cys2-Cys4, and Cys3-Cys6 and its NMR-derived solution structure contains what authors described as a 'double-turn' motif [27, 28]. This is different than the 'triple-turn' motif described for M-1 branch peptide tx3a [4, 29]. Thus, even though the disulfide connectivity pattern is the same for these two peptides, their secondary structures vary considerably. Prior to this discovery, it was thought that once a structure was determined for a peptide family with the same disulfide pattern, the peptide scaffold was conserved for that family of peptides. The unique secondary structure characteristics of mr3e and tx3a are suspected to be responsible for the observed difference in response to intracranial injection in mice with conotoxin mr3e causing no observable effect and tx3a inducing excitatory behavior [30]. This excitatory behavior is also observed for the M-2 branch peptide, mr3a [28]. The mr3a structure consists of the 'triple-turn' motif also observed for tx3a, but its backbone has a different cystine pattern. Figure 2 shows a comparison of the published structures, disulfide bond pattern, and sequences for mr3e, tx3a, and mr3a [27].

-Figure 2-

M-2 Branch: There is limited published information describing members of the M-2 branch of the M-superfamily. The structure and sequence of conotoxin mr3a have been reported, but little is known about biological targets or action mechanisms for this and other M-2 peptides. Conotoxin mr3a was determined to have a cystine connectivity of Cys1-Cys6, Cys2-Cys4, and Cys3-Cys5 [28, 30] and a three dimensional structure best described as a 'triple-turn' motif [4]. To date, this connectivity belongs exclusively to the M-2 branch peptides. Additionally, there appear to be similarities between the M-2 branch and the Maxi-M peptides. A resemblance between the prepropeptide sequences for the immature conotoxins has been reported. The M-2 and M-4/M-5 branches have a common-size signal peptide (25 residues) in addition to a high degree of homology in the first eight residues for that signal peptide, MMSKLGVL [30]. It has further been described that the M-2 peptide mr3a elicits a strong excitatory response in mice at nanomolar quantities upon intracranial injection [4]. This finding supports the notion that members of the M-2 branch peptides are significant and functional snail venom components with the potential to be used as a means of discovering the possible existence of uncharacterized receptors.

M-3 Branch: The cystine pattern and structural characteristics of M-3 branch peptides have not been reported to date. Current knowledge of these peptides is limited to sequence comparisons with other branch families of the M-superfamily. A study exploring similarities to other families noted that both M-1 and M-3 peptides are rich in acidic residues, as opposed to basic residues in M-2 and M-4 [30]. cDNA studies were performed to further investigate the similarities between the M-1 and M-3 branches. From this, a new phylogenetic tree was constructed in which M-1 and M-3 conotoxins have "a closer relationship, falling in the same tree branch" [30]. The signal peptide and pro-peptide region of the immature toxins appear to be highly conserved; evidence that the M-1 and M-3 branch peptides are evolutionarily related. This close relationship does not appear between any other branch families in the M-superfamily [30]. A few recently reported examples of M-3 peptides are: reg12a (GCCOOQWCGODC**TSO**CC) [1], Tx3.5 (RCCKFPCPDSC**RYL**CC(nh2)) [26], and Qc3.1 (ACCDPDWCDAGC**YDG**CC) [27]. Most M-3 family peptides are discovered by PCR amplification of cDNA [27]. Peptide sequencing allows for characterization into the M-3 branch based on the cysteine pattern and three amino acid residues in the third loop.

Maxi-M's: The Maxi-M peptides are divided into the M-4 and M-5 branches of the M-superfamily based on their primary sequence. These peptides are further separated into the κM-, ψ-, and μ-conotoxins based on their molecular targets consisting of potassium, nicotinic acetylcholine, and sodium receptors, respectively [4, 5]. The M-4 branch consists of all published κM- and ψ- conotoxins in addition to about one third of the μ-conotoxins, (Figure 3) while the M-5 branch consists of only μ-conotoxins to date. It is also interesting to note that the Maxi-M, κM-, ψ-, and μ-conotoxins are found mostly in fish-hunting *Conus* species, whereas the Mini-M peptides appear in the venom of the mollusk and worm-hunting cone snails [28, 29]. The Maxi-M peptides induce paralysis in prey, while the M-1, M-2, and M-3 branch (Mini-M) peptides generally cause an excitatory physiological response [4]. This difference may be attributed to the feeding preferences of the snail. The fish-hunting cone snails paralyze their fast moving prey before consumption, while worm- or snail-hunting cone snails rely on spasmodic response to drive the prey from its shelter. Characteristic differences for the Maxi-M peptides versus the Mini-M's can be summarized as: a longer primary sequence (22-24 a.a. vs <20), a known molecular target (K^+, nACh, Na^+ receptors), and a common and distinctive cystine pattern (Cys1-Cys4, Cys2-Cys5, Cys3-Cys6) [27, 31-33]. Until the more recent characterization of the Mini-

M peptides, it was believed that the Maxi-M disulfide bond pattern was the archetype of the entire M-superfamily; a commonality observed in other conotoxin superfamilies. It has recently been demonstrated that the disulfide bonds are significant structural and electrostatic elements involved in the interaction of conotoxins with the ligand binding domain of receptor ion channels [34]. The electrostatic properties of the sulfur atoms in the disulfide bonds contribute to conotoxin agonist and antagonist activity toward a class of biological receptors that have recently been classified as the Cys-loop superfamily of receptors [35].

-Figure 3-

The Cys-loop superfamily receptors have been found in nicotine acetylcholine receptors and play a role in the transmission of nerve impulses, specifically in central and peripheral nervous tissue, and neuromuscular junctions, which control muscle contraction [36]. The structure of the Cys-loop receptors is largely comprised of β-sheets and α-helices responsible for the transmission of action potentials across cell membranes. It is supposed that the α-helical region of the Cys-loop receptors is responsible for the initiation of the action potential [35]. Regulation of these receptors is vital to the well-being of the organism; and it appears that conotoxins have evolved to target this class of receptors.

M-4 Branch: The M-4 branch is defined by 4 residues in the third loop region of the primary sequence between Cys4 and Cys5 ($CC(X_{4\text{-}6})C(X_{4\text{-}5})C(\mathbf{X_4})CC$). The M-4 branch κM- and ψ-conotoxins were the first conotoxins for which a disulfide bond pattern was determined and ψ-PIIIE was the first M-superfamily peptide for which a three dimensional structure was determined by NMR spectroscopy. Thus the M-4 peptides serve as a structure model for all ψ-conotoxins.

κM-Conotoxins: κM-conotoxins are antagonists of K^+ ion channels. In conotoxin nomenclature, the κ refers to activity toward K^+ ion channels and the M indicates that the peptide belongs to the M-superfamily. The only published κM-conotoxin at this time is κM-RIIIK, with the primary sequence: LOS**C**SLNLRL**C**OVOA**C**KRNO**CC**T(nh2). This peptide was isolated and characterized from the venom of *C. radiatus* [31]. Its biological target was determined to be the "*Shaker*" channel and the similar mammalian $K_v1.2$ K^+ channel [33]. The "Shaker" channel was first identified from *Drosophila*. Applied stimulus to induce rapid muscle contraction caused the fly to "shake" [37-39]. The inhibition of the channel protein responsible for the shaking affect was then selectively inhibited confirming the existence of what is now referred to as the "shaker" channel. This channel was further classified as a potassium voltage-gated channel allowing for the identification of subsequent similar channels in numerous species. κM-RIIIK has the highest demonstrated affinity for TSha1 (Trout Shaker Channel 1), a *Shaker* K^+ channel from rainbow trout (*Onchorychus mykiss*) [33, 31, 40]. In comparison with the O-superfamily conotoxin, κ-PVIIA, [41-42] and other venom components targeting the *Shaker* channel, κM-RIIIK appears to function uniquely; it does not have the characteristic κ-conotoxin functional dyad consisting of hydrophobic amino acids [32]. The proposed mechanism of κ-conotoxins is to use the hydrophobic dyad structure to plug the *Shaker* channel pore. In contrast, κM-RIIIK inhibits the *Shaker* channel by forming a ring that occludes the channel pore by acting as a surface lid rather than an intercalating plug in the channel [31-33, 42]. The amino acids essential for the K^+ channel binding of κM-RIIIK appear to be: Leu1, Arg10, Lys18, and Arg19 [32]. The significance of these amino acids was identified based on mutational analysis comparison with μ-conotoxin GIIIA [32]. The structure and function characterization of κM-RIIIK provides a new backbone model on which future pharmacological treatments may be derived.

ψ-Conotoxins: The M-4 branch of the M-superfamily contains three examples of ψ-conotoxins that have been labeled ψ-PIIIE, ψ-PIIIF, and ψ-PrIIIE [43-45]. These ψ-conotoxins contain the same disulfide connectivity as the rest of the M-4 branch [43]. Similar to the κ- and κM- association, ψ-conotoxins function at the nicotinic acetylcholine receptors (nAChRs) that were previously determined to be the site of activity for the αA-conotoxins [43-47]. αA-Conotoxins are much smaller (<20 amino acids) and only contain two disulfide bonds [44].

In 1997, ψ-PIIIE became the first ψ-conotoxin to be described in literature. This peptide was isolated from the venom of *C. purpurascens* and was reported to have the amino acid sequence: HOO**CC**LYGK**C**RRYOG**C**SSAS**CC**QR(nh2) [43]. It was further discovered that *C. purpurascens* inhibits muscle nAChRs using a combination of ψ-PIIIE and αA-PIVA [43]. The binding of ψ-PIIIE to nAChRs in muscle was

determined to occur at a ligand binding site complementary to that identified for αA-conotoxins [43, 46-47]. Significant to the discovery of ψ-PIIIE was the identification of the novel ligand binding site on muscle nAChRs. Thus, ψ-PIIIE served as a useful tool to characterize a new target for therapeutic drug development, and increase understanding of the Cys-loop superfamily of receptors.

ψ-PIIIF was reported in 2003, a component of the venom of *C. purpurascens* with the sequence: GOO**CC**LYGS**C**ROFOG**C**YNAL**CC**RK(nh2) [45]. Though also a ψ-conotoxin, PIIIF is ~50 times less potent than ψ-PIIIE in blocking both mice and elasmobranch nAChRs [45]. The difference in functionality is thought to be due to the amino acids of the third loop region. In ψ-PIIIE the third loop consists primarily of small polar residues (SSAS), while in ψ-PIIIF the third loop consists of bulkier residues (YNAL) that collectively form a less polar region of the peptide. The variation in amino acid composition has led researchers to hypothesize that polarity and steric bulk in the third loop are the significant factors for the observed deviation in binding and activity of these peptides to nAChRs [45].

ψ-Conotoxin PrIIIE isolated from the venom of *Conus parius* has the sequence, AAR**CC**TYHGS**C**LKEK**C**RRKY**CC**(nh2) [44]. ψ-PrIIIE is approximately 28 times more potent than ψ-PIIIE at inhibiting nAChRs (IC_{50} = ~250 nM for ψ-PrIIIE as compared to ~7,000 nM for ψ-PIIIE) [44]. Whereas ψ-PIIIE-F, and ψ-PrIIIE all inhibit fish nAChRs, only ψ-PrIIIE is active as a non-competitive inhibitor of mice skeletal muscle nAChRs [44]. This is significant as the mouse model is used to predict thereputic activity in humans.

M-4 μ-Conotoxins: μ-Conotoxins belong to both the M-4 and M-5 branches of the M-superfamily. These conotoxins differ only in the number of residues in the third loop region and are similar in their common activity as antagonists of voltage-gated sodium channels (VGSCs). VGSCs control the electron potential across neuron and excitable muscle cell membranes [48]. These VGSCs are $Na_v1.1$ – $Na_v1.9$ based on their α-subunit sequence and further organized according to their sensitivity to tetrodotoxin (TTX). VGSCs are classified as either TTX-S, for sensitive, and TTX-R, for resistant, depending on their overall degree of inhibition by tetrodotoxin [49-50]. All nine VGSCs are inhibited by TTX but those with IC_{50} values in the nanomolar range constitute the TTX-S VGSCs ($Na_v1.1$-4, $Na_v1.6$, and $Na_v1.7$), and the VGSCs that have IC_{50} values in the micromolar range are considered TTX-R ($Na_v1.5$, $Na_v1.8$ and $Na_v1.9$) [34, 48-49, 51-55]. The TTX-S VGSCs function in either neuronal communication ($Na_v1.1$, $Na_v1.2$-3, and $Na_v1.6$-7) [49, 56] or skeletal muscle control ($Na_v1.4$) [48-49]. Researchers have determined the connection between VGSCs and the symptoms caused by many diseases including neuropathic pain, arrhythmia, epilepsy, stroke, and bipolar disorder [48, 57]. For example, neuropathic pain originates from the TTX-R channels $Na_v1.8$ and $Na_v1.9$ [58-60].

μ-Conotoxins have been investigated as therapeutic drugs because of their potency and ability to differentiate between distinct VGSCs. The first μ-conotoxins isolated from the venom of *Conus geographus* were μ-GIIIA, B, and C. These peptides were shown to be active in blocking skeletal muscle VGSCs [54], specifically $Na_v1.4$, while having a much lower affinity toward neuronal VGSCs. The activity of conotoxin μ-GIIIA toward the inhibition of $Na_v1.4$ VGSCs was shown to be dependent on Arg^{13} in the primary sequence (R in bold): RDCCTOOKKCKD**R**QCKOQRCCA-(nh2) [54, 61]. Substitution of this Arg residue (Arg^{13}) three amino acids removed from the third Cys residue (i.e. loop two) results in the inactivation of the peptide. This is a testament to the specificity and usefulness of conotoxins as tools to understand ligand to receptor activity.

More recent discoveries have led to the identification, characterization, and publication of 18 μ-conotoxins to date. The 7 μ-conotoxins belonging to the M-4 branch are: GIIIA-C [49, 37], PIIIA [49, 51], TIIIA [49], and SxIIIA-B [62]. The remaining 11 μ-conotoxin belong to the M-5 branch: SIIIA [48, 55], KIIIA [55], CIIIA, CnIIIA-B, MIIIA [63], SIIIB [58], SmIIIA [64], and BuIIIA-C [12]. μ-Conotoxin PIIIA blocks both muscle and neuronal TTX-S VGSCs [53, 56, 65]. μ-Conotoxin PIIIA was found to have an IC_{50} value of 2 μM in tests involving mammalian CNS Type II muscle channels [53]. This represents an inhibition that is upwards of 50 times greater that that observed for μ-conotoxin GIIIA [51]. μ-PIIIA is also effective in blocking neuronal channels ($Na_v1.2$) *albeit* with lower affinity than muscle VGSCs [63]. μ-PIIIA has a pIC_{50} of 6.8 (where $pIC_{50} = -\log IC_{50}$) in binding studies with $Na_v1.4$ skeletal muscle VGSCs [37, 52, 66-67]. While $Na_v1.4$ skeletal muscle VGSCs serve as the model system for muscle cell antagonist activity, the $Na_v1.2$ VGSCs serve as the standard test receptor for neuronal VGSCs (Figure 4).

-Figure 4-

More recently, μ-conotoxin TIIIA was isolated from the venom of *Conus tulip*. The primary sequence for this peptide contains the characteristic and functionally relevant positively charged basic amino acid Arg (or Lys), three amino acids removed from the third Cys residue (i.e. loop two), next to an acidic and negatively charged amino acid (Glu^{15}) [49]. This is unusual as the sequences of previous μ-conotoxins contained the Arg (or Lys) neighbored by either Gln, Arg, or Trp [49]. μ-TIIIA inhibits VGSCs from both neuronal ($Na_v1.2$) and skeletal muscle ($Na_v1.4$), with a preference for $Na_v1.4$ VGSCs. Mutation of Glu^{15} to Ala in the TIIIA peptide, changed the biological target preference from skeletal muscle $Na_v1.4$ VGSCs to neuronal $Na_v1.2$ [49]. This is an example of the value of conotoxins as selective probes to develop structure activity relationship models for closely related receptors. Single-site mutation on the conotoxin scaffold provided a better understanding of the size and electrostatic topography of the ligand required to differentiate between receptor types.

μ-Conotoxins SxIIIA and SxIIIB demonstrate high affinity for muscle $Na_v1.4$ VGSCs [62]. While little has been described in the literature regarding these recently reported μ-conotoxins, the process used to identify their disulfide connectivity made use of a novel technique referred to as rapid disulfide bridge mapping [62]. This procedure introduces isotope labeled cysteine residues during cloning, then uses nuclear magnetic resonance (NMR) spectroscopy to identify the connectivity of the labeled peptides and rapidly determine bridge order. When synthesizing SxIIIA, the first three cysteines were uniformly ^{15}N and ^{13}C enriched, while the remaining three cysteine residues were labeled with a 70:30 mix of ($^{14}N/^{12}C$):($^{15}N/^{13}C$) [62]. The use of NMR to differentiate the cystine pattern is an efficient approach that may lead to the characterization of many additional conotoxins that are so far known only by sequence because of insufficient native sample quantities for confirmation of correct folding of synthetic peptide.

M-5 Branch: The M-5 branch of the M-superfamily differs from those preceding it by containing five residues in the third cysteine loop between cysteines four and five. All known M-5 conotoxins are μ-conotoxins [27].

M-5 μ-Conotoxins: The μ-conotoxins within the M-5 branch are: SmIIIA [64], SIIIA [48, 55], KIIIA [55], CIIIA, CnIIIA-B, MIIIA [63], SIIIB [58] and BuIIIA-C [12].). In contrast to the M-4 branch μ-PIIIA and μ-GIIIA that inhibit TTX-S VGSCs, μ-SmIIIA is an antagonist of TTX-R VGSCs [64]. Although SmIIIA inhibits TTX-R channels, it does not differentiate well between them ($Na_v1.5$, $Na_v1.8$, and $Na_v1.9$) [66].

μ-Conotoxins SIIIA and KIIIA are antagonists of TTX-R channels similar to the M-4 peptide μ-SmIIIA. Interestingly, the size of the first loop varies between μ-SmIIIA (five amino acids) to μ-SIIIA (three residues) and finally μ-KIIIA (one residue) [48, 55]. The diversity in the number of amino acids in the first loop was an indication to researchers that the second and third loop regions contained the necessary structural and electrostatic characteristics for the peptides to be active toward inhibiting TTX-R VGSCs. This was confirmed in a study that removed the first loop of μ-KIIIA and showed that the remainder of the peptide retained efficacy toward the inhibition of TTX-R VGSCs. The modified μ-KIIIA provided a smaller yet equally effective model from which synthetic mimetics are being explored as pharmacological therapeutics [34].

Based on sequence comparison from the last two loop regions of μ-SmIIIA, KIIIA, and SIIIA, four new μ-conotoxins, CnIIIA-B, CIIIA, and MIIIA were discovered and reported in 2006 [63]. As expected, these more recent μ-conotoxins inhibited TTX-R VGSCs. Despite their sequence similarity to one another, they were found to inhibit a divergent set of TTX-R channels, indicating the importance of the amino acid constituents within these peptides. Thus, it was determined that the secondary structures of the second and third loop regions of μ-SmIIIA, KIIIA, and SIIIA are significant for binding to TTX-R channels. This was tested by a comparison study of these three conotoxins with μ-CnIIIA, μ-CIIIA, and μ-MIIIA, which yielded a consensus sequence of $CC(X_n)CSX^{R}_{K}WCRDH^{A}_{S}{}^{R}_{Q}CC$ indicating the three significant amino acids present in the second and third loops, that are responsible for differentiating between the TTX-R channels [63]. Common to these μ-conotoxins is the conservation of Arg in the second loop, usually in the same sequence position (third residue past Cys) [37, 48-49, 51, 55, 58, 62-64], further validating the significance of Arg in VGSC inhibition first reported for μ-GIIIA.

A similar effect, as reported with μ-TIIIA, was observed for μ-conotoxins SIIIA and SIIIB where μ-SIIIA differs from μ-SIIIB by a single residue change in the third loop that results in SIIIB preferentially targeting $Na_v1.4$ (muscle) to $Na_v1.2$ (neuron), while SIIIA prefers neuronal VGSCs [58].

The M-superfamily peptides have advanced the methods used to identify novel peptides belonging to the M-5 branch based on exogene analysis. A study by Mandë Holford at the University of Utah presented the structure of a new sodium channel blocking conotoxin extracted from *C. bullatus*, BuIIIA [12], which also shows an affinity for muscle VGSCs $Na_v1.4$. μ-BuIIIA was identified and characterized by the isolation of exogenes which are the genes responsible for the mediation of biotic interactions between organisms [12]. Once isolated, these genes were compared with the genes of different cone snail species. The phylogenetic information resultant from this gene comparison approach was incorporated into a general strategy aimed at the discovery of new classes of peptides [12]. This technique led to the discovery of the *C. bullatus* μ-conotoxins, BuIIIA, BuIIIB, and BuIIIC. It is thought that this approach to conotoxin detection will expedite the characterization and reporting of novel peptides previously not identifiable by traditional means. After all, it was the sequence comparison of many M-superfamily peptides containing five amino acids in the third loop that resulted in the M-5 branch appearing in the literature in 2009.

Uncharacterized M-superfamily conotoxins: A new branch of the M-superfamily has been described in the literature that is homologus in signal sequence to other M-superfamily branches, but differs in cysteine order. The two conotoxins in this new category are Vx2 and Im6.1 [9, 15]. A study performed at the Shanghai Institute of Biological Sciences led to the publication of conotoxin Vx2. Their report demonstrated the commonality between cDNA and mRNA for conotoxin Vx2 and the M-4 branch peptides μ-GIIIA and μ-GIIIB. Out of the 25 amino acid residues comprising the signal sequences for Vx2 and the μ-GIIIA-C there was only a difference in two residues: Leu9 was substituted by Val; and Met23 was replaced by Leu [9]. These differences are considered to be within an acceptable margin of variance to allow Vx2 to be characterized as an M-superfamily peptide. However, experts in the field disagree with this assignment because of the unique cysteine order in mature Vx2 (Table 1). M-superfamily peptides all have the characteristic cysteine arrangement of CC-C-C-CC while that of Vx2 is CCC-C-C-C [9].

Conotoxin Im6.1, isolated from the venom of *Conus imperialis*, was placed in the same neoteric category of the M-superfamily as Vx2. Like Vx2, Im6.1 shows a similar cDNA and mRNA signal sequence consistent with other members of the M-superfamily. However, Im6.1 has the cysteine arrangement of C-C-CC-C-C with the disulfide connectivity C1-C4, C2-C5, and C3-C6 [15]. The biological targets of Vx2 and Im6.1 are yet to be identified.

-Table 1-

Conclusion: The M-superfamily of conotoxins is arguably the most diverse of all the conotoxin superfamilies yet characterized. Five branches of this family have been definitively categorized with conotoxins Vx2 and Im6.1 demonstrating signal sequences consistent with the M-superfamily, yet with unique cysteine and cystine patterns. The study of the M-superfamily has been pivotal in increasing understanding of conotoxins and their specific receptors. Through study of the Maxi-M conotoxins, the μ-, κM-, and ψ-conotoxins have been characterized with their respective targets (Na^+, K^+, and nAChR). The Maxi-M peptides that constitute the M-4 and M-5 branches have defined disulfide connectivity and known target receptors.

The smaller Mini-M conotoxins of the M-1 and M-2 branches not only differ in cystine order form the Maxi-M peptides, but also from each other. This is unique to the M-superfamily and has led to the interesting discovery that conotoxins with different cystine pattern can have similar structures even when they belong to different branches of the superfamily. This is observed with the structures of the M-1 peptides mr3e and tx3a having dissimilar structure, while the M-2 peptide mr3a has a similar three dimensional structure to the M-1 peptide tx3a. This serves as another unique feature to the M-superfamily. The uncharacterized disulfide pattern, unknown molecular target, and yet to be determined structural information for M-3 peptides offer many potential rewards for researchers.

Although much remains unknown regarding the M-superfamily of peptides, especially the Mini-M branches, the scientific advancement that has occurred through the study of these peptides has been significant. The study of Maxi-M conotoxins has involved the use of original techniques leading to the advancement in structure activity

relationship comprehension for neuronal and muscular molecular targets. Research on the M-superfamily peptides has led to the development of new instruments, probes, and laboratory techniques that are broadly applicable to other fields of science. A specific example is the use of isotopic enrichment of cysteine side chains to characterize cystine arrangement by NMR. This technique can be applied to any disulfide rich peptide or protein to reduce the time required for structure determination and connectivity elucidation. Secondly, the use of exogene phylogeny comparison can be used across the genus *Conus* to identify and characterize peptides that would otherwise not be possible by traditional means.

The M-superfamily of conotoxins has displayed unique characteristics that have allowed for the development of therapeutic treatments and increased knowledge of receptors responsible for pain and sensory transmission. In summary, there remains much work to be done in this field.

Acknowledgments
We would like to thank Drs. Greg Bulaj and Kenneth A. Cornell for their contributions to this manuscript. Additional thanks are owed to Seth Eidemiller, Steven D. Jacob, Matthew Turner, Luke Woodbury, and Aubrey Johnston for their editorial input. This publication was made possible by NIH Grant #P20 RR016454 from the INBRE Program of the National Center for Research Resources, Mountain States Tumor Medical Research Institute, and Research Corporation Cottrell College Scholars Program.

FIGURE LEGENDS

Figure 1. A flowchart of the organization of the M-superfamily with known disulfide connectivity shown for each branch (M-1 – M-5).

Figure 2. Structures of Mini-M peptides of the M-1 and M-2 branches. Depicted are the 'Double-turn' motif of mr3e (M-1) and the 'Triple-turn' of tx3a (M-1) and mr3a (M-2).

Figure 3. Structures of the M-4 branch sub-categories κM-, μ-, and ψ-conotoxins. Explanations of highlighted residues are described in the text.

Figure 4. Known binding efficacy for μ-conotoxins toward muscle voltage gated sodium channels (VGSCs) and Neuronal VGSCs.

TABLE LEGEND

Table 1. A summary of known M-superfamily branches, cysteine patterns, disulfide arrangements, representative sequences, three dimensional structures, and references.

Figure 1

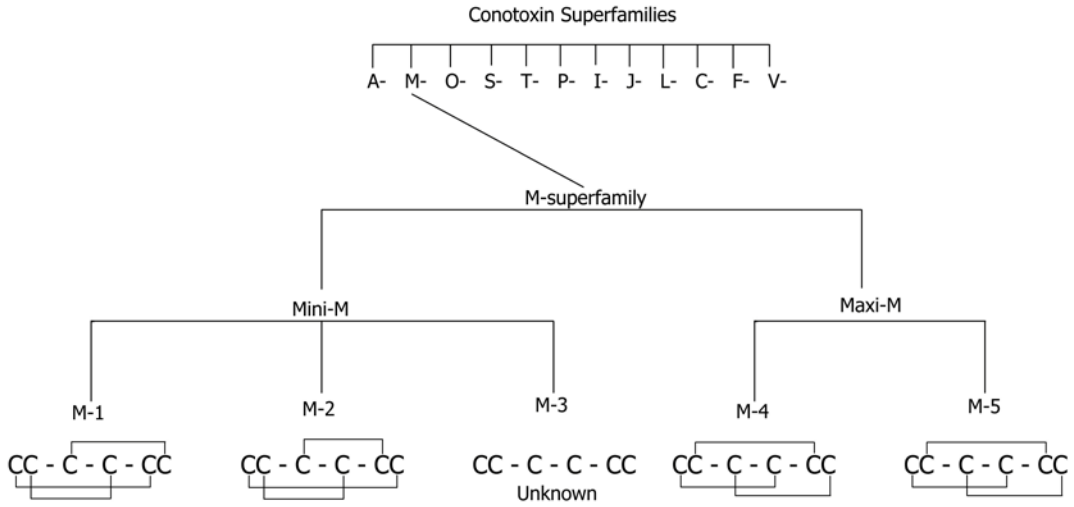


Figure 2

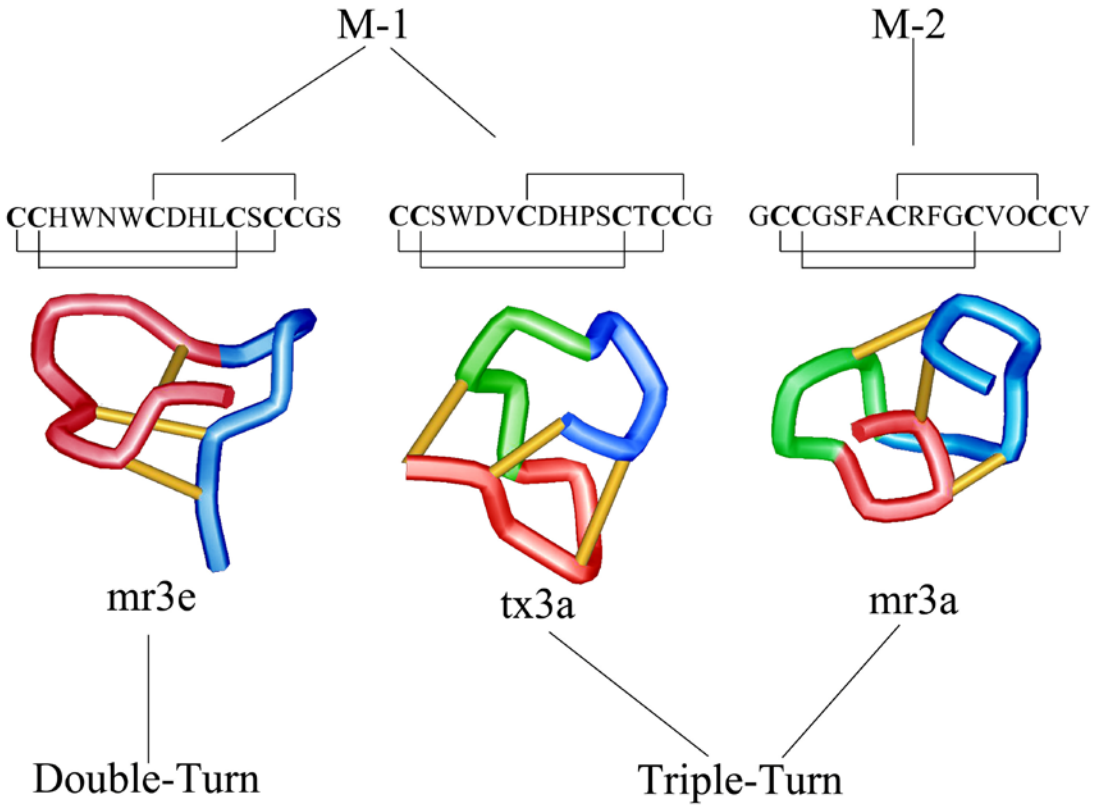


Figure 3

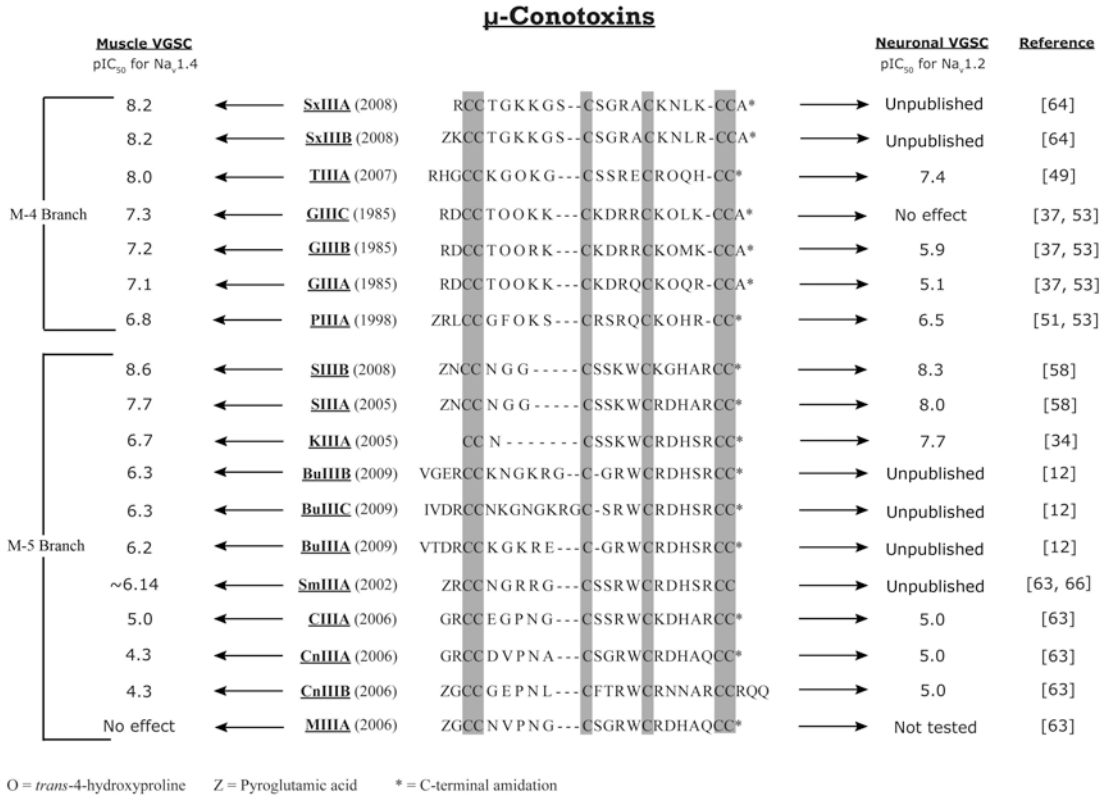


Figure 4

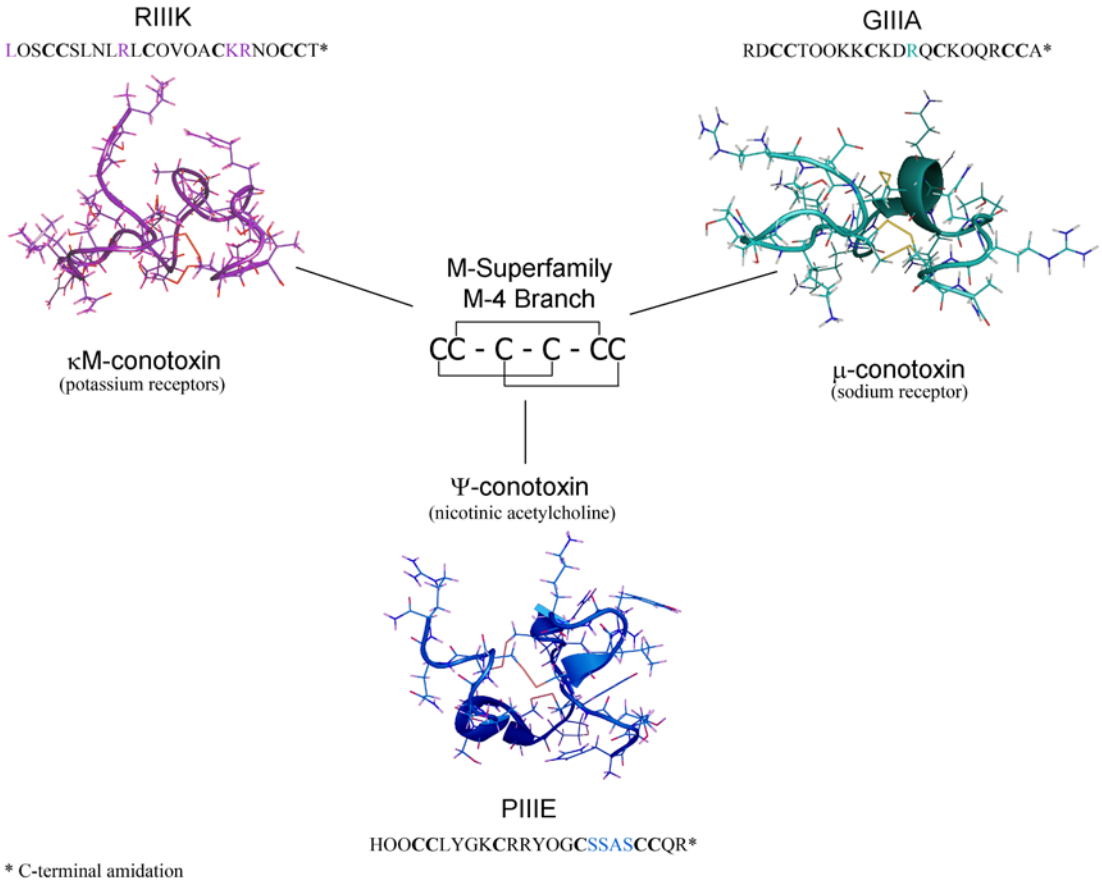
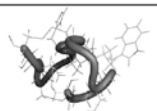
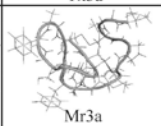
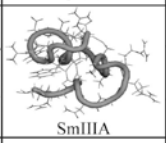


Table 1

M-Superfamily						
Mini-M	M-1	CC - C - C - CC	Tx3a	CCSWDVCDHPSCTCCG	 Tx3a	4, 15
			Lp3.1	CCEPQWCDGACDCCS		27, 30, 15
			Mr3e	VCCPFGGCHELCYCCD*		27, 28, 30, 15
	M-2	CC - C - C - CC	Mr3a	GCCGSFACRFGCVOCCV	 Mr3a	29, 15
			reg12g	CCMALCSRYHCLPCC		1, 15
			Tx3b	CCPPVACNMGCKPCC*		26, 15
	M-3	CC - C - C - CC Unknown	Reg12a	GCCOOQWCGODCTSOCC	Unknown Reg12a	1, 15
			Vn3.4	GCCEPDWCDSGCDDGCC		15
			Tx3.5	RCCKFPCPDSCRYLCC*		26, 15
Maxi-M	M-4	CC - C - C - CC	GIIIA	RDCCTOOKKCKDRQCKOQRCCA*	 GIIIA	14, 15
			TIIIA	RHGCCKGOKGCSSRECROQHCC*		15
			RIIIK	LOSCCSLNLRLCOVOACKRNOCCT*		33, 15
	M-5	CC - C - C - CC	SmIIIA	ZNCCNGGCSSKWCRDHARCC*	 SmIIIA	12, 34, 15
			KIIIA	CCNCSSKWCRDHSRCC*		12, 34, 15
			BuIIIA	VTDRCCKGKRECGRWCRDHSRCCGRR		12
	Unknown	CCC - C - C - C Unknown	Vx2	WIDPSHYCCCGGGCTDDCVNC	Unknown Vx2	9, 15
		C - C - CC - C - C	Im6.1	TCDPYYCNDGKVCCPEYPTCGDSTGKLICVRVTD	Unknown Im6.1	15

O = *trans*-4-hydroxyproline Z = Pyroglutamic acid * = C-terminal amidation

REFERENCES

1. Franco, A., Pisarewicz, K., Moller, C., Mora, D., Fields, G. B., Mari, F. (2006) Hyperhydroxylation: A new strategy for neuronal targeting by venomous marine molluscs. Prog. Mol. Subcell. Biol. 43, 83-103.
2. Myers, R. A., Cruz, L. J., Rivier, J. E., and Olivera, B. M. (1993) Conus peptides as chemical probes for receptors and ion channels. Chem. Rev. 93(5), 1923-1936.
3. Kohn, A. J., Nishi, M., and Pernet, B. (1999) Snail Spears and Scimitars: A Character Analysis of *Conus* Radular Teeth. J. Moll. Stud. 65, 461-481.
4. McDougal, O. M., Turner, M. W., Ormond, A. J., and Poulter, C. D. (2008) Three-dimensional structure of conotoxin tx3a: an m-1 branch peptide of the M-superfamily. Biochemistry 47, 2826-2832.
5. Olivera, B. M., and Cruz, L. J. (2001) Conotoxins, in retrospect. Toxicon 39, 7-14.
6. Kohn, A. J. (1956) Piscivorous gastropods of the genus *Conus*. Proc. Natl. Acad. Sci. USA 42, 168-171.
7. Spence, I., Gillessen, D., Gregson, R. P., and Quinn, R. J. (1997) Characterization of the neurotoxic constituents of *Conus geographus* (L) venom. Life Sci. 21, 1759-1770.
8. Clark, C., Olivera, B. M., and Cruz, L. J. (1981) A toxin from *Conus geographus* venom which acts on the vertebrate central nervous system. Toxicon 19, 691-699.
9. Jiang, H., Wang, C. Z., Xu, C. Q., Fan, C. X., Dai, X. D., Chen, J. S., and Chi, C. W. (2006) A novel M-superfamily conotoxin with a unique motif from *Conus vexillum*. Peptides 27, 682-689.
10. Olivera, B. M. (1997) *Conus* venom peptides, receptor and ion channel targets and drug design: 50 million years of neuropharmacology. Mol. Biol. Cell 8, 2101-2109.
11. Norton, R. S. and Olivera, B. M. (2006) Conotoxins down under. Toxicon 48, 780-798.
12. Holford, M., Zhang, M. M., Gowd, K. H., Azam, L., Green, B. R., Watkins, M., Ownby, J. P., Yoshikami, D., Bulaj, G., and Olivera, B. M. (2009) Pruning nature: biodiversity-derived discovery of novel sodium channel blocking conotoxins from *Conus bullatus*. Toxicon 53, 90-98.
13. McDougal, O. M. (1998) *Conus* peptides investigated by NMR spectroscopic methods. (Doctoral dissertation, University of Utah).
14. Yao, S., Zhang, M. M., Yoshikami, D., Azam, L., Olivera, B. M., Bulaj, G., and Norton, R. S. (2008) Structure, dynamics, and selectivity of the sodium channel blocker (mu)-conotoxin SIIIA. Biochemistry 47, 10940-10949.
15. Kass, Q., Westermann, J. C., Halai, R., Wang, C. K., and Craik D. J. (2008) ConoServer, a database for conopeptide sequences and structures. Bioinformatics 24(3), 445-446.
16. Mehdiratta, R., and Saberwal, G. (2007) Bio-business in brief: the case of conotoxins. Current Science 92(1), 39-45.
17. Sandall, D. W., Satkunanathan, N., Keays, D. A., Polidano, M. A., Liping, X., Pham, V., Down, J. G., Khalil, Z., Livett, B. G., Gayler, K. R., (2003) A novel alpha-conotoxin identified by gene sequencing is active in suppressing the vascular response to selective stimulation of sensory nerves in vivo. Biochemistry 42, 6904-6911.
18. Adams, D. J., Smith, A. B., Schroeder, C. L., Yasuda, T., Lewis, R. (2003) ω-conotoxin CVID inhibits a pharmacologically distinct voltage-sensitive calcium channel associated with transmitter release from preganglionic nerve terminals. J. Biol. Chem. 278, 4057–4062.

19. Alonso, D., Khalil, Z., Stakunanthan, N., and Livett, B. G., (2003) Drugs from the sea: conotoxins as drug leads for neuropathic pain and other neurological conditions. Mini Rev. Med. Chem. 3, 785-787.
20. Bowersox, S. S., and Luther, R. (1998) Pharmacotherapeutic potential of omega-conotoxin MVIIA (SNX-111), an N-type neuronal calcium channel blocker found in the venom of *Conus magus*. Toxicon 36(11), 1651–1658.
21. Atanassoff, P. G., Hartmannsgruber, M. W., Thrasher, J., Wermeling, D., Longton, W., Gaeta, R., Singh, T., Mayo, M., McGuire, D. and Luther, R. R. (2000) Ziconotide a new N-type calcium channel blocker, administered intrathecally for acute postoperative pain. Reg. Anesth. Pain Med. 25, 274-278.
22. Penn, R. D., and Paice, J. A. (2000) Adverse effects associated with the intrathecal administration of ziconotide. Pain 85(1-2), 291-296.
23. Levin, T., Petrides, G., Weiner, J., Saravay, S., Multz, A. S., and Bailine, S. (2002) Intractable delirium associated with ziconotide successfully treated with electroconvulsive therapy. Psychosomatics 43, 63-66.
24. Malmberg, A. B., Gilbert, H., McCabe, R. T., and Basbaum, A. I. (2003) Powerful antinociceptive effects of the cone snail venom-derived subtype-selective NMDA receptor antagonists conantokins G and T. Pain 101, 109-116.
25. Skov, M. J., Beck, J. C., de Kater, A. W., Shopp, G. M. (2007) Nonclinical safety of ziconotide: an intrathecal analgesic of a new pharmaceutical class. Int. J. Toxicol. 26(5), 411-421.
26. Corpuz, G. P., Jacobsen, R. B., Jimenez, E. C., Watkins, M., Walker, C., Colledge, C., Garrett, J. E., McDougal, O., Li, W., Gray, W. R., Hillyard, D. R., Rivier, J., McIntosh, J. M., Cruz, L. J., Olivera, B. M. (2005) Definition of the M-conotoxin superfamily: characterization of novel peptides from molluscivorous *Conus* venoms. Biochemistry 44, 8176-8186.
27. Han, Y. H., Wang, Q., Jiang, H., Liu, L., Xiao, C., Yuan, D. D., Shao, X. X., Dai, Q. Y., Chemng, J. S., and Chi, C. W. (2006) Characterization of novel M-superfamily conotoxins with new disulfide linkage. FEBS Journal 273, 4972-4982.
28. Du, W. H., Han, Y. H., Huang, F. J., Li, J., Chi, C. W., and Fang, W. H. (2007) Solution structure of an M-1 conotoxin with a novel disulfide linkage. FEBS Journal 274, 2596-2602.
29. McDougal, O. and Poulter, C. D. (2004) Three-dimensional structure of the Mini-M conotoxin mr3a. Biochemistry 43, 425-429.
30. Wang, Q., Jiang, H., Han, Y. H., Yuan, D. D., and Chi, C. W. (2008) Two different groups of signal sequence in M-superfamily conotoxins. Toxicon 51, 813-822.
31. Ferber, M., Sporning, A., Jeserich, G., DeLaCruz, R., Watkins, M., Olivera, B. M., and Terlau, H. (2003) A novel *Conus* peptide ligand for K+ channels. J. Biol. Chem. 278(4), 2177-2183.
32. Al-Sabi, A., Lennartz, D., Ferber, M., Gulyas, J., Rivier, J. E. F., Olivera, B. M., Carlomagno, T., and Terlau, H. (2004) kappaM-conotoxin RIIIK, structural and functional novelty in a K^+ channel antagonist. Biochemistry 43(27), 8625-8636.
33. Verdier, L., Al-Sabi, A., Rivier, J. E. F., Olivera, B. M., Terlau, H., and Carlomagno, T. (2005) Identification of a novel pharmacophore for peptide toxins interacting with K+ channels. J. Biol. Chem. 280(22), 21246-21255.

34. Khoo, K. K., Feng, Z. P., Smith, B. J., Zhang, M. M., Yoshikami, D., Olivera, B. M., Bulaj, G., and Norton, R. S. (2009) Structure of the analgesic (mu)-conotoxin KIIA and effects on structure and function of disulfide deletion. Biochemistry 48, 1210-1219.
35. Sine, S. M., and Engel, A. G. (2006) Recent advances in Cys-loop receptor structure and function. Nature 440, 448-455.
36. Lester, H. A., Dibas, M. I., Dahan, D. S., Leite, J. F., and Dougherty, D. A. (2004) Cys-loop receptors: new twists and turns. Trends Neurosci. 27(6), 229-336.
37. Cruz, L. J., Gray, W. R., Olivera, B. M., Zeikus, R. D., Kerr, L., Yoshikami, D., and Moczydlowski, E. (1985) *Conus geagraphus* Toxins that discriminate between neuronal and muscle sodium channels. J. Biol. Chem. 260(16), 9280-9288.
38. Hodgkin, A. L., and Huxley, A. F. (1952) A quantitative description of membrane current and its application to conduction and excitation in nerve. J. Physiol. (Lond.). 117, 500-544.
39. Catterall, W. A. (1984) The molecular basis of neuronal excitability. Science 233, 653-661.
40. Ferber, M., Al-Sabi, A., Stocker, M., Olivera, B. M., and Terlau, H. (2004) Identification of a mammalian target of κM-conotoxin RIIIK. Toxicon. 43, 915-921.
41. Shon, K-J., Stocker, M., Terlau, H., Stuhmer, W., Jacobsen, R., Walker, C., Grilley, M., Watkins, M., Hillyard, D. R., Gray, W. R., and Olivera, B. M. (1998) kappa-Conotoxin PVIIA is a peptide inhibiting the shaker K+ channel. J. Biol. Chem. 273(1), 33-38.
42. Jacobsen, R. B., Koch, E. D., Lange-Malecki, B., Stocker, M., Verhey, J., Van Wagoner, R. M., Vyazovkina, A., Olivera, B. M., and Terlau, H. (2000) Single amino acid substitutions in kappa-conotoxin PVIIA disrupt interaction with the shaker K+ channel. J. Biol. Chem. 275(32), 24639-24644.
43. Shon, K. J., Grilley, R. J., Cartier, G. E., Hopkins, C., Gray, W. R., Watkins, M., Hillyard, D. R., Rivier, J., Torrres, J., Yoshikami, D., and Olivera, B. M. (1997) A noncompetitive peptide inhibitor of the nicotinic acetylcholine receptor from *Conus purpurascens* venom. Biochemistry 36(31), 9581-9587.
44. Lluisma, A. O., Lopez-Vera, E., Bulaj, G., Watkins, M., and Olivera, B. M. (2008) Characterization of a novel psi-conotoxin from *Conus parius*. Toxicon 51, 174-180.
45. Van Wagoner, R. M., Jacobsen, R. B., Olivera, B. M., and Ireland, C. M. (2003) Characterization and three-dimensional structure determination of psi-conotoxin PIIIF, a novel noncompetitive antagonist of nicotinic acetylcholine receptors. Biochemistry 42(21), 6353-6362.
46. Mitchell, S. S., Shon, K. J., Foster, M. P., Davis, D. R., Olivera, B. M., and Ireland, C. M. (1998) Three-dimensional solution structure of conotoxin psi-PIIIE, and acetylcholine gated ion channel antagonist. Biochemistry 37(5), 1215-1220.
47. Van Wagoner, R. M., and Ireland C. M., (2003) An improved solution structure for psi-conotoxin PIIIE. Biochemistry 43(21), 6347-6352.
48. Wang, C-Z., Zhang, H., Jiung, H., Lu, W., Zhao, Z-Q., Chi, C-W. (2006) A novel conotoxin from *Conus striatus*, mu-SIIIA, selectively blocking rat tetrodotoxin-resistant sodium channels. Toxicon 47, 122-132.
49. Lewis, R. J., Schroeder, C. I., Ekberg, J., Nielson, K. J., Loughnan, M., Thomas, L., Adams, D. A., Drinkwater, R., Adams, D. J., and Alewood, P. F. (2007) Isolation and structure-activity of mu-conotoxin TIIIA, a potent inhibitor of tetrodotoxin-sensitive voltage-gated sodium channels. Mol. Pharmacol. 71, 676-685.

50. Catterall, W. A., Goldin, A. L., and Waxman, S. G. (2005) International Union of Phamacology. XLVII. Nomenclature and structure-function relationships of voltage-gated sodium channels. Pharmacol. Rev. 57, 397-409.
51. Shon, K. J., Olivera, B. M., Watkins, M., Jacobsen, R. B., Gray, W. R., Floresca, C. Z., Cruz, L. J., Hillyard, D. R., Brink, A., Terlau, H., Yoshikami, D. (1998) mu-Conotoxin PIIIA, a new peptide for discriminating among tetrodotoxin-sensitive Na channel subtypes. J. Neurosci. 18(12), 4473-4481.
52. Safo, P., Rosenbaum, T., Shcherbatko, A., Choi, D. Y., Han, E., Toledo-Aral, J. J., Olivera, B. M., Brehm, P., and Mandel, G. (2000) Distinction among neuronal subtypes of voltage-activated sodium channels by mu-conotoxin PIIIA. J. Neurosci. 20(1), 76-80.
53. Nielsen, K. J., Watson, M., Adams, D. J., Hammarstrom, A. K., Gage, P. W., Hill, J. M., Craik, D. J., Thomas, L., Adams, D., Alewood, P. F., and Lewis, R. J. (2002) Solution structure of mu-conotoxin PIIA, a preferential inhibitor of persistent TTX-sensitive sodium channels. J. Biol. Chem. 277, 27247-27255.
54. Nakamura, M., Niwa, Y., Ishida, Y., Kohno, T., Sato, K., Oba, Y., and Nakumura, H. (2001) Modification of Arg-13 of μ-conotoxin GIIIA with piperidinyl-Arg analogs and their relation to the inhibition of sodium channels. FEBS Letters 503, 107-110.
55. Bulaj, G., West, P. J., Garrett, J. E., Watkins, M., Zhan, M-M., Norton, R. S., Smith, B. J., Yoshikami, D., and Olivera, B. M. (2005) Novel conotoxins from *Conus striatus* and *Conus kinoshitai* selectively block TTX-resistant sodium channels. Biochemistry. 44(19), 7259-7265.
56. Goldin, A. L., Barchi, R. L., Caldwell, J. H., Hofmann, F., Howe, J. R., Kallen, R. G., Mandel, G., Meiseler, M. H., and Netter, Y. B. (2000) Nomenclature of voltage-gated sodium channels. Neuron 28, 365-368.
57. Clare, J. J., Tate, S. N., Nobbs, M., and Romanos, M. A. (2000) Voltage-gated sodium channels as therapeutic targets. Drug Discov. Today 5, 506-520.
58. Schroeder, C. I., Ekberg, J., Nielsen, K. J., Adams, D., Loughnan, M. L., Thomas, L., Adams, D. J., Alewood, P. F. and Lewis, R. J. (2008) Neuronally mu-conotoxins from *Conus striatus* utilize an alpha-helical motif to target mammalian sodium channels. J. Biol. Chem. 283, 21621-21628.
59. Kalso, E. (2005) Sodium channel blockers in neuropathic pain. Curr. Pharm. Des. 11, 3005-3011.
60. Wood, J. N., Boorman, J. P., Okuse, K., and Baker, M. D. (2004) Voltage-gated sodium channels and pain pathways. J. Neurobiol. 61, 55-71.
61. Sato, K., Ishida, Y., Wakamatsu, K., Kato, R., Honda, H., Ohizumi, Y., Nakamura, H., Ohya, M., Lancelin, J. M., and Kohda, D. (1991) Active site of mu-conotoxin GIIIA, a peptide blocker of muscle sodium channels. J. Biol. Chem. 266, 16989-16991.
62. West, P. J., Bulaj, G., Garrett, J. E., Olivera, B. M., and Yoshikami, D. (2002) mu-Conotoxin SmIIIA, a potent inhibitor of tetrodotoxin-resistant sodium channels in amphibian sympathetic and sensory neurons. Biochemistry. 41(51), 15388-15393.
63. Zhang, M. M., Fiedler, B., Green, B. R., Catlin, P., Watkins, M., Garrett, J. E., Smith, B. J., Yoshikami, D., Olivera, B. M. and Bulaj, G. (2006) Structural and functional diversities among mu-conotoxins targeting TTX-resistant sodium channels. Biochemistry 45, 3723-3732.
64. Walewska, A., Skalicky, J. J., Davis, D. R., Zhang M-M., Lopez-Vera, E., Watkins, M., Han, T. S., Yoshikami, D., Olivera, B. M., and Bulaj, G. (2008) NMR-based mapping of

disulfide bridges in cysteine-rich peptides: application to the mu-conotoxin SxIIIA. J. Am. Chem. Soc. 130(43), 14280-14286.
65. Catteral, W. A. (2000) From ionic currents to molecular mechanisms: the structure and function of voltage-gated sodium channels. Neuron 26, 13-25.
66. Keizer, D. W., West, P. J., Lee, E. F., Yoshikami, D., Olivera, B. M., Bulaj, G., and Norton, R. S. (2003) Structural basis for tetrodotoxin-resistant sodium channel binding by mu-conotoxin SmIIIA. J. Biol. Chem. 278(47), 46805-46813.
67. Moczydlowski, E., Olivera, B. M., Gray, W. R., and Strichartz, G. R. (1986) Discrimination of muscle and neuronal Na-channel subtypes by binding competition between saxitoxin and p-conotoxins. Proc. Natl. Acad. Sci. U.S.A. 83, 5321-5325.