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Review

Marine Toxins That Target Voltage-gated Sodium Channels

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Abstract: Eukaryotic, voltage-gated sodium (Na_V) channels are large membrane proteins which underlie generation and propagation of rapid electrical signals in nerve, muscle and heart. Nine different Na_V receptor sites, for natural ligands and/or drugs, have been identified, based on functional analyses and site-directed mutagenesis. In the marine ecosystem, numerous toxins have evolved to disrupt Na_V channel function, either by inhibition of current flow through the channels, or by modifying the activation and inactivation gating processes by which the channels open and close. These toxins function in their native environment as offensive or defensive weapons in prey capture or deterrence of predators. In composition, they range from organic molecules of varying size and complexity to peptides consisting of ~10-70 amino acids. We review the variety of known Na_V -targeted marine toxins, outlining, where known, their sites of interaction with the channel protein and their functional effects. In a number of cases, these natural ligands have the potential applications as drugs in clinical settings, or as models for drug development.

Keywords: voltage-gated sodium channel; marine neurotoxins; sodium channel receptor sites.

Abbreviations: AneTx, anemone toxin (often abbreviated ATX in other papers); ATx, antillatoxin; B toxins, ribbon worm toxins; Ca_V , Na_V , and K_V , voltage-gated ion channels selective for calcium, sodium or potassium, respectively; NMDA, *N*-methyl-D-aspartate; CigTx, ciguatoxin; CLA, clathrocin; CNS, central nervous system; PNS, peripheral nervous system; DBS, dibromosceptin; EC_{50} , concentration producing 50% of the maximal effect;

GPT, *Goniopora* (coral) toxin; KTx, kalkitoxin; IC₅₀, concentration producing 50% inhibition; NSP, neurotoxic shellfish poisoning; LD₅₀, lethal dose/concentration for 50% of population; K_d, dissociation constant; OTx, ostreotoxin; PbTx-n, brevetoxins where n = 1-10; PSP/PST, paralytic shellfish poisoning/toxins; STx, saxitoxin; TTx, tetrodotoxin; TTx-s, TTx sensitive; TTx-r, TTx resistant.

1. Overview

In this chapter, we broadly sketch the range of marine toxins that interact with voltage-gated sodium (Na_v) channels, outlining their functional effects and discussing their actions in the context of recognized ligand binding sites on the channel. Four other articles in this issue provide more specialized treatments of different groups of toxins: tetrodotoxin resistance and adaptive mutations in targeted species (Geffeny and Ruben), ciguatoxins (Nicholson and Lewis), and conotoxins (Layer and McIntosh). We organize our presentation in terms of functional effects of the toxins, such as pore blockers and gating modifiers, and within these functionally circumscribed groups, we consider both peptide and non-peptide agents. Generally, the non-peptides are smaller metabolites, which are concentrated in food chains that may span from bacteria to vertebrates, and become of particular concern for human health and economic reasons during the phytoplankton blooms commonly known as “red tides” [139]. In contrast, the peptides of interest generally are larger molecules, which are elaborated in several phyla of eukaryotes, in specialized structures such as nematocysts or venom glands. The identification and classification of the Na_v channel receptors, through which both peptide and non-peptide toxins and modulators act, has progressed through definitions based strictly on functional interactions to, in some cases, identification of specific amino acid residues and atomic interactions involved. Where possible, we present our overview of functional groups of Na_v channel targeted toxins in the context of the relevant, recognized receptors on the channel.

2. Structure of Na_v channels

Eukaryotic Na_v channels are integral membrane proteins consisting of a main structural α subunit of ~2000 amino acids [22-24], and one or more β subunits [70; 71]. Na_v channels are members of a family of voltage-gated ion channels which also includes calcium (Ca_v) and potassium (K_v) channels. The voltage-gated channels are members of a larger superfamily of P loop channels, which include the smaller inwardly rectifying K⁺ channels. The simplest members of the voltage-gated channel family are homotetrameric, and include a number of delayed rectifier K_v channels and certain prokaryotic Na_v channels [108; 118]. Homotetrameric channels are presumably the evolutionary predecessors of the 4-domain members of the voltage-gated channel family.

The α subunit of Na_v channels (Figure 1) is made up of four homologous domains, I-IV, each of which possesses six transmembrane helices, S1-S6, with cytoplasmic N- and C-termini. The cytoplasmic, III-IV connecting loop forms the fast inactivation gate of the channel [122]. Each of the four major domains can be sub-divided into a voltage sensing domain (S1-S4) and a pore-forming P

domain (S5-S6). Within each P domain, the S5-S6 connector loops into the membrane to form, in sequence from its N- to C- terminal end, a random-coiled “turret”, short supporting P helix, the lining outer pore including the narrowest part – the selectivity filter, and a P-S6 connector. The P domain forms part or all of the receptors for a number of pore-blocking toxins and drugs, including guanidinium toxins and μ -conotoxins. The voltage-sensor domain includes a conspicuous S4 segment, with Arg or Lys residues, spaced generally at every third position, and comprising most of the charges contributing to gating charge movement generated during voltage-sensitive channel gating. A complementary acidic residue in S2 also makes some contribution to gating current [128]. Channel opening following voltage activation appears to occur by a conformational change near the crossing of S6 helices towards the cytoplasmic end of the conducting pore. Inactivation occurs on several time scales: fast – gated by the III-IV linker latching to a receptor near the cytoplasmic end of the S6 segments, and a complex of slow inactivation processes which appear, at least, to involve conformational changes in the selectivity filter near the outer end of the pore [147; 149]. Na_V channels typically show more than 10-fold selectivity for Na^+ ions over other physiological ion species [63], based on a narrow selectivity filter [63], whose primary element is a ring of residues, DEKA [126], contributed by the DI-DIV respectively, arranged clockwise as viewed from the extracellular end of the conducting pore [37; 84].

Figure 1. Schematic presentation of the membrane topology of the α subunit of an Na_V channel. Homologous domains (DI-DIV), and, within each domain, transmembrane segments (S1-S6) are labeled. Each domain has a P loop (P) between S5-S6 segment and a cationic, voltage-sensing (+) S4 segment. The hydrophobic sequence (IFM) in the intracellular DIII-DIV connecting loop is required for fast inactivation, and serves as inactivation latch for the Na^+ channel. Representative Na_V channels have been cloned from different tissues including: rat brain (rNav1.2), rat skeletal muscle (rNav1.4), adult rat heart (rNav1.5), and PNS (rNav1.9). Residue numbering for each domain is indicated.

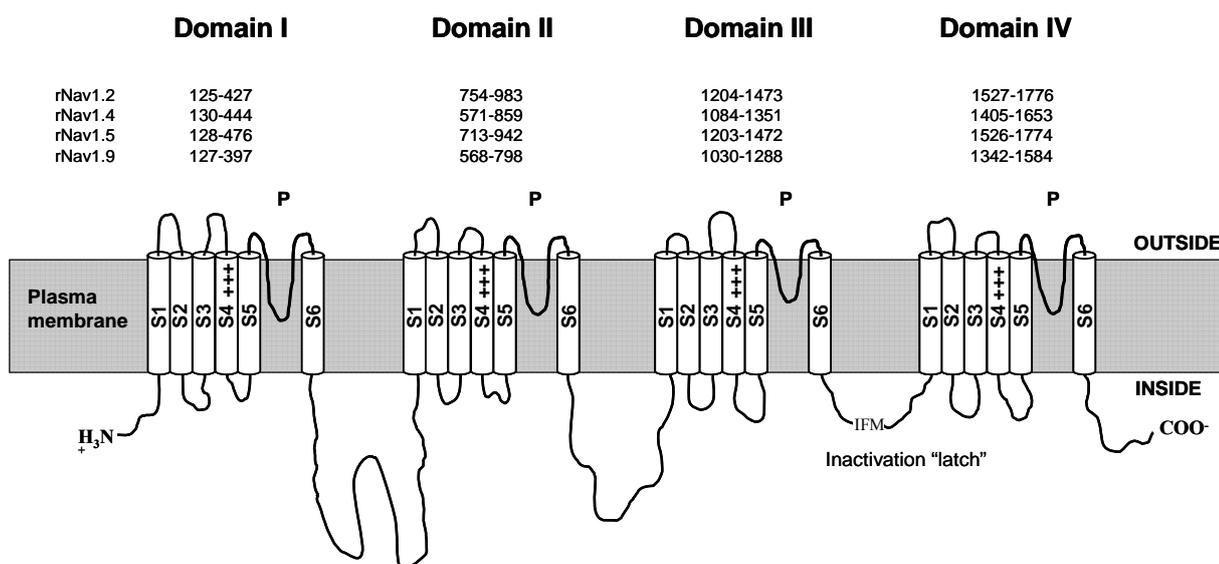


Table 1: Neurotoxins that target the Na_v channels and their corresponding receptor sites.

Receptor Site	Physiological Effect	Putative location	Toxin	Organism
1	Pore blocker	P loop at DI, DII, DIII, DIV	Tetrodotoxins Saxitoxins μ -Conotoxins Batrachotoxin Veratridine	Puffer fish (Tetraodontiformes), starfish, molluscs, tunicates, dinoflagellates, symbiotic bacteria Shellfishes, Red tide dinoflagellates and symbiotic bacteria Cone snails
2	Persistent activation gating; Depolarization of resting potentials Repetitive firings	DI-S6 DII-S6 DIII-S6 DIV-S6	Aconitine Grayanotoxin	[Dendrobatid “poison dart” frogs] [In the seed of <i>Schoenocaulon officinale</i> and in rhizome of <i>Veratrum album</i>] [In the root of <i>Aconitum</i> plants] [In plants of the family <i>Ericaceae</i>]
3	Prolonged Na ⁺ channel opening	DIV:S3-S4 DI:S3-S4 Loop	α -Scorpion toxins Sea anemone toxins	[Scorpion <i>spp.</i>] Sea anemones <i>spp.</i>
4	Inactivation gating modifier Agonist Shifts in activation gating; repetitive firings	DIV:S5-S6 Loop ? DII:S3-S4 Loop	δ -Atracotoxins Antillatoxin β -Scorpion toxins	[Funnel-web spider <i>Hadronyche versuta</i>] Dinoflagellate <i>Lyngbya majuscula</i> [Scorpion <i>spp.</i>]
5	Shift in activation gating	DI:S6 DIV:S5	Brevetoxins Ciguatoxins	Red tide dinoflagellate <i>Karenia brevis</i> Red tide dinoflagellate <i>Gambierdiscus toxicus</i> and tropical coral fishes
6	Prolonged Na ⁺ channel opening	DIV:S3-S4	δ -Conotoxins	Cone snails
7	Inhibitor, not a pore blocker Persistent activation gating; depolarization of resting potentials; repetitive firings	? DI-S6 DII-S6 DIII-S6	Kalkitoxin DDT and analogs, Pyrethroids	Dinoflagellate <i>Lyngbya majuscula</i> [Insecticide chemicals]
8	Inactivation gating modifier Prolonged Na ⁺ channel opening	? ?	Goniopora Conus striatus toxin	genus <i>Goniopora</i> <i>Conus striatus</i>
9	Inhibition of Na ⁺ permeability	DI-S6 DIII-S6 DIV-S6	Local anesthetics, Anticonvulsants, Antidepressants	[Chemicals from various sources]
N.I.	Inhibitor, not a pore blocker	?	μ O-Conotoxins	Cone snails
N.I.	Inactivation gating modifier	?	B-Toxins	Ribbon worm (<i>Cerebratulus lacteus</i>)
N.I.	Inhibitor, not a pore blocker	?	Meroditerpenoids	Brown algae
N.I.	Inactivation gating modifier	?	Clathrocin	Sponge (genus <i>Agelas</i>)
N.I.	Inactivation gating modifier	?	Dibromosceptrin	Sponge (genus <i>Agelas</i>)
N.I.	Antagonist	?	Jamaicamides A-C	Dinoflagellate <i>Lyngbya majuscula</i>
N.I.	Inactivation gating modifier	?	Ostreotoxin-3	Dinoflagellate <i>Ostreopsis lenticularis</i>

Note: Site 1-9 after references [27; 151; 167]. Neurotoxins with unknown binding receptor sites are categorized as N.I. (Not identified). Neurotoxins not originating from marine organisms are mentioned within square brackets [].

3. Drug and toxin receptors on Na_v channels

Beginning in the 1960s and 1970s with the pioneering studies of Narahashi, Moore, Catterall and colleagues, ligand sites on Na_v channels have been identified and classified. Key pharmacological targets in Na_v channels tend to be clustered on the functionally important structures, which are intimately associated with activation and inactivation gating, and with ion conduction and selectivity. Nine receptors are widely recognized, as listed in Table 1.

In Figure 2, we loosely map the nine receptor sites on to a cartoon derived from the recent crystal structure of the voltage-gated K⁺ channel, K_v1.2, in Figure 2. The K_v1.2 structure [90] is the only available, high resolution structure of a voltage-gated eukaryotic channel, and thus provides the most up to date clues as to the likely structure of the four-domain Na_v and Ca_v channels. In the discourse which follows, we collect the different toxins into functional groups, each of which is cross-referenced to the receptor site with which it interacts, where this is known.

4. Na_v channel blockers and other inhibitors

4.1. Receptor site 1

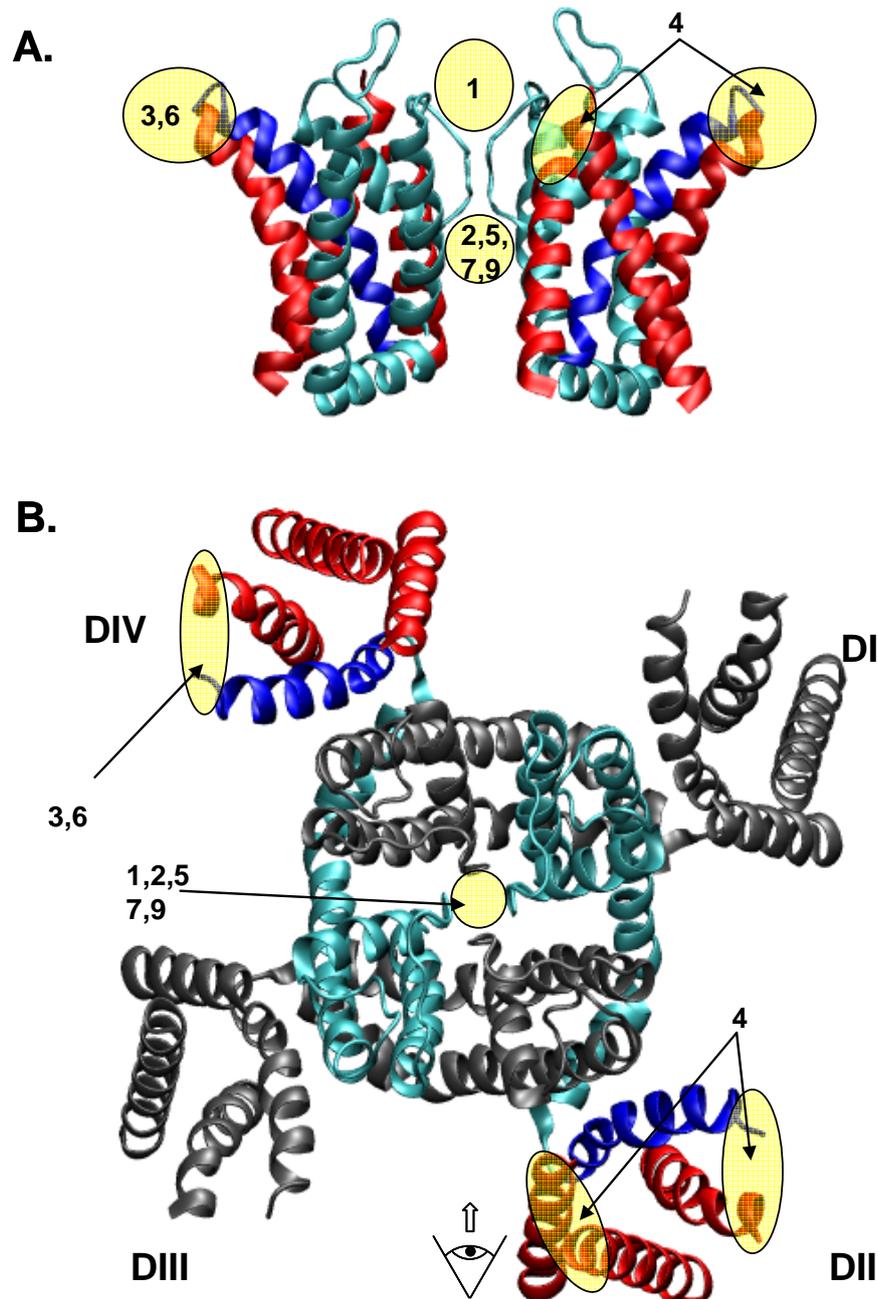
We will define as pore “blockers” toxins which inhibit ion transport by sterically and/or electrostatically occluding the ion conducting pore of the channel. The best known Na_v pore blockers are tetrodotoxin (TTx), saxitoxin (STx) and the μ -conotoxins (see below). Receptor site 1 is located in the outer vestibule of the channel and is formed by the P loops from the four domains of the channel (see Figs. 1 and 2). Historically, the functional criterion for a toxin or drug to be considered to act at site 1 has been competition for binding with TTx or STx. The similar functional effects of chemically different blockers notwithstanding, the specific channel residues that make the most important contacts with different toxins and drugs will depend on the size and structure of the blocker. Thus, smaller heterocyclic toxins such as TTx and STx have less points of interaction with the pore than larger peptide molecules like the μ -conotoxins, which have a more complex set of contacts between ligand and receptor. The strongest known interactions of all of these toxins are in the vestibule-lining P loops within 3 or 4 residues on the C-terminal side of the residues making up the DEKA “inner ring” of the selectivity filter. Particularly important for binding of the GIII μ -conotoxins are the “outer ring” acidic residues located 3 (domains I, II, and IV) or 4 (domain III) residues C-terminal from the DEKA ring. In addition, extrapore residues have been shown to affect both affinity and selectivity among different channel isoforms [28; 85]. Identified interactions of μ -conotoxin GIIIA have been recently reviewed in detail by French and Terlau [52].

Studies on rat brain channels identified the following domain I residues as important for high affinity TTx block: D384, E387, Y385 [100; 143]. Here, we cite rNa_v1.2 numbering, in which the inner DEKA ring is made up of D384, E942, K1422, and A1714. A somewhat different set of channel residues provided the most important interactions for STx. These included E942 and E945 (domain II), and K1422 and D1426 (domain III) [143]. A more recent, detailed analysis of interactions of TTx and STx with Na_v1.4 has been provided by Dudley and colleagues [29; 30; 110; 111].

Different channel isoforms from brain, heart and muscle have a different sensitivity to TTx, STx, and to μ -conotoxins. In TTx sensitive (TTx-s) channels in brain and adult skeletal muscle, an aromatic residue appears essential for high affinity block by TTx. In skeletal muscle (rNa_v1.4), this is Y401, corresponding to F385 in a rat brain isoform (rNa_v1.2), while the homologous residue in the relatively TTx-resistant (TTx-r) heart channel is C374 (rNa_v1.5), as reviewed by Denac and coworkers [35]. A similar amino acid, located in the P loop regions, is associated with TTx sensitivity in different neuronal channels. In dorsal root ganglia neurons, F385 is found in TTx-s Na_v channels, while C or S is present in the homologous position in TTx-r channels, [104]. An earlier study found that mutation of E403Q in the rat skeletal muscle sodium channel, which essentially abolishes TTx and STx block, reduced sensitivity to μ -conotoxin GIIIA and GIIIB only about 4-fold [137].

Thus, different toxins may produce similar pharmacological effects by interacting with different subsets of residues within the same larger surface on the target channel molecule. The key to selective targeting of drugs may lie in a deeper understanding of the non-overlapping portions of larger receptor sites. Ecologically, Na_v channel blockers may play either defensive or offensive roles [120].

Figure 2. Nav channel receptor sites mapped onto the structure of Kv1.2. **A.** Side-view of Kv1.2 with P loops modified to resemble more closely the likely structure of Nav channel P loops, which allow block by agents such as STx, TTx, and μ -conotoxin. Only DII and DIV shown; S1-S3 red, S4 blue and S5-P loop-S6 cyan. **B.** Top view of Kv1.2 showing all four domains with domains DII and DIV colored as in A. The eye at the bottom shows the point of view for part A. The figures were produced using VMD software [68] and final images rendered with POV-ray (<http://www.povray.org/>).



4.2. Non-peptide Na_V channel pore blockers and inhibitors

4.2.1. Tetrodotoxin

Tetrodotoxin is one of the best known marine toxins because of health concerns based on its involvement in fatal food poisoning, its unique chemical structure, and its specific action of blocking Na_V channels of excitable membranes [27; 64; 164; 167]. The toxin, whose name was derived from the order of fish with which it is most commonly associated, the Tetraodontiformes. There are more than 40 species of tetraodon pufferfish, known in Japan as fugu [27]. Other marine organisms, including polyclad flatworms [120], and some terrestrial poisonous animals, have also been found to store TTx. However, bony fish from the family Tetraodontidae are especially well known to cause tetrodotoxin poisoning. These fish do not possess a venom apparatus, but TTx may be found in all organs of the body. The occurrence and distribution of TTx among different phylogenetic lines of organisms gave evidence for speculation that TTx and its derivatives originated from symbiotic microorganisms. Indeed, a number of bacteria, including the genera *Aeromonas* and *Alteromonas*, *Escherichia coli*, *Photobacterium phosphoreum*, *Plesiomonas shigelloides*, *Pseudomonas sp.* and some *Vibrio sp.* have been shown to produce TTx (reviewed in [51; 79; 164]).

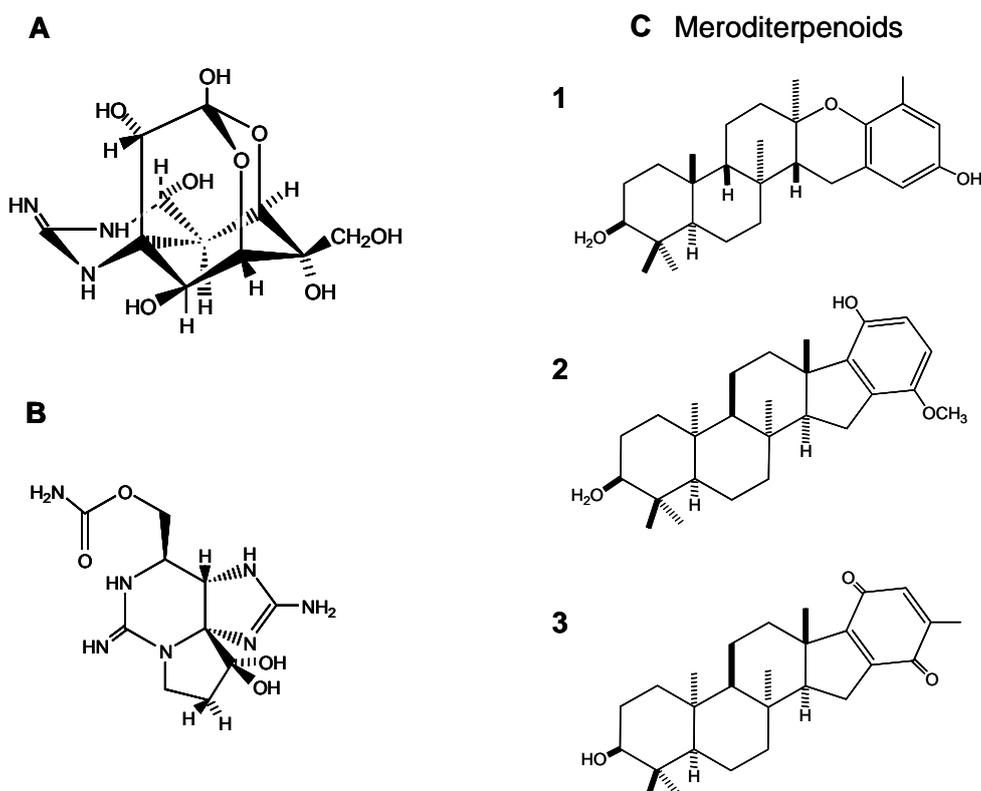
TTx is of amphoteric nature with an unusual tricyclic structure (Figure 3-A). It possesses both cationic (guanidine) and anionic (acid hydroxyl) groups. TTx binds with high affinity [dissociation constant (K_d) in the nM concentration range] to the external surface of Na_V channels by binding to site 1 of these channels [27]. The positively charged guanidinium group interacts with a negatively charged carboxyl group at the mouth of the Na_V channel on the extracellular side of plasma membranes of excitable membranes. When it does so, the inward flow of Na^+ through the channel pore is blocked, leading to inhibition of action potential generation and propagation [64; 79]. Seven derivatives of TTx have been detected in puffer fish, newts and poisonous frogs. These TTx derivatives vary in their potencies [164]. The value of this particular toxin in electrophysiological research is profound. Recent models of the molecular structure of the Na_V pore have been strongly based on interactions with this toxin, and Na_V channels are often divided broadly into two pharmacological classes, TTx-s and TTx-r.

4.2.2. Saxitoxin

Paralytic shellfish poisoning (PSP) is a deadly affliction that results from the accidental consumption of some potent natural neurotoxins, typically via contaminated seafood [114]. Among all seafood poisoning, PSP poses the most serious threat to public health, and the economic damage caused by accumulation of toxins in shellfish is immeasurable (see [79] and references therein). Saxitoxin (STx) and its analogs are collectively called paralytic shellfish toxins (PST) and are considered the causative agents for PSP. The name STx was derived from the mollusc in which it was first identified, *Saxidomus giganteus* [79]. Toxins are present in dinoflagellates such as *Alexandrium sp.*, *Pyrodinium bahamense var. compressum* and *Gymnodinium catenatum* [88; 135] during red tides, which causes STx to be concentrated by filter-feeding shellfish and subsequently, to be conveyed to humans when they consume the shellfish. Neurological symptoms occur shortly after the time of ingestion, and, in extreme cases, can lead into death [166]. Although it has been generally considered

that PST are associated with dinoflagellates, there is evidence that heterotrophic bacteria are responsible for toxin synthesis in these organisms [31; 55; 92; 148].

Figure 3. Chemical structures of non-peptide Na_V channels blockers. **A.** Tetrodotoxin, and **B.** Saxitoxin, both of which are tricyclic, guanidinium-derived marine toxins. **C.** Meroditerpenoids from the marine brown alga *Styopodium flabelliforme*, which act as Na_V channel modifiers: **1.** 2 β ,3 α -Epitaondiol; **2.** Flabellinol; **3.** Flabellinone. The structures of the meroditerpenoids were reproduced from Sabry et al. [123], with permission.



The saxitoxins are a family of water-soluble neurotoxins and are among the most potent toxins known. Information on the toxicity of saxitoxins is mainly restricted to acute toxicity in mammals and humans (reviewed in [79]). For example, the LD_{50} values (lethal dose/concentration for 50% of population) of STx by intravenous, intraperitoneal and oral routes in mice are 2.4 $\mu\text{g}/\text{kg}$, 10 $\mu\text{g}/\text{kg}$ and 263 $\mu\text{g}/\text{kg}$, respectively. Saxitoxins are tricyclic compounds (Figure 3-B) and their molecular skeletons are structurally related to TTx, but with two guanidinium moieties [131] in STx compared to the one guanidinium moiety in TTx. More than 20 analogs have been described [79]. Like the case of TTx, saxitoxins are well documented non-peptide neurotoxins that are highly selective blockers of Na_V channels in excitable cells at site 1, thereby affecting nerve and muscle impulse generation in animals [27; 64; 131; 164; 167]. Recently a new, exceptionally potent derivative of saxitoxin has been described [165].

4.2.3. Meroditerpenoids

Brown algae (Phaeophyceae) produce a variety of secondary metabolites possessing many different structural varieties and biological activities ([50] and reviews therein). The genus *Styopodium* is a tropical group of the Phaeophyceae well-known for its rich complement of polycyclic diterpenoids fused to oxidized aromatic rings (meroditerpenoids) [50]. These terpenoid metabolites are useful chemotaxonomic markers because of their structural complexity and genetic origins [56]. Meroditerpenoids display potent biological activities, with toxic and enzyme-modulating functions, which may be of biomedical and pharmacological utility [155]. Three new meroditerpenoids, 2 β ,3 α -epitaondiol (Figure 3-C-1), flabellinol (Figure 3-C-2) and flabellinone (Figure 3-C-3), from *Styopodium flabelliforme* were assayed to have neurotoxic and Na_v channel modulation activity [123]. In this study, these compounds were cytotoxic to NCI-H460 cells with an LD₅₀ of 24, 9, and 14 μ M, respectively. In a Na_v channel modulation assay using the neuro-2a neuroblastoma cell line, with STx as a control inhibitor, these compounds showed detectable Na_v channel blocking activity at 0.7, 2, and 7 μ M, respectively. Overall, these meroditerpenoid metabolites show a broad range of biological activities, including being toxic to fish and brine shrimp, and to some human cancer cells. Some of this toxicity may be due to a blocking of Na_v channels [50; 123].

4.3. Peptide Na_v channel pore blockers and other inhibitors

4.3.1. μ -Conotoxins

Conotoxins in general, and μ -conotoxins in particular, are found in the venom of predatory tropical marine cone snails, genus *Conus*. Venom is a primary weapon used by these molluscs to capture prey (small fish or invertebrates) and to defend themselves. Venoms of all cone snails are complex and an individual species may express 100-200 venom peptides. Toxic components of the venom are mostly small peptides [106], which are synthesized in a tubular venom duct. All cone snails have specialized radular teeth that penetrate the skin of prey and inject venom. These teeth work as a harpoon and sharp needles. After the venom injection, the prey becomes quickly paralyzed and is now available for consumption. Some fish-hunting species (e.g., *Conus geographus*) are dangerous to humans, and fatalities have been recorded in the medical literature [144].

μ -Conotoxins are highly basic, cysteine-rich peptides which have three disulphide bridges in the three loop framework. The amino acid sequences of seven μ -conotoxins showed in Figure 4, and some basic properties of these μ -conotoxins are displayed in Table 2.

The number of recognized μ -conotoxins is rapidly increasing, thanks to a combination of molecular cloning techniques to identify new sequences, and application of improved synthetic procedures to produce oxidized, active conformations of the folded toxins (see Table 3). NMR-derived 3D structures of GIIIA, PIIIA and SmIIIA are shown in Figure 5. Discovery of new toxins is revealing increased functional diversity in the μ -conotoxin family. In addition, recent work shows that there is a divergence of the disulfide framework generating structural diversity among the μ -conotoxins [32]. At present time, no μ -conotoxins are used directly in a clinical application. However, discovery of new isoform-specific μ -conotoxins from *Conus* venoms, or constructs not existing in nature, offers a promising way to neurologically active drugs [1]. Discoveries of μ -conotoxins SmIIIA, SIIIA and

KIIIA open new opportunities for creation of specific drugs for pain relief. These toxins block the TTx-r Nav channels that are responsible for pain signal propagation in dorsal root ganglia neurons.

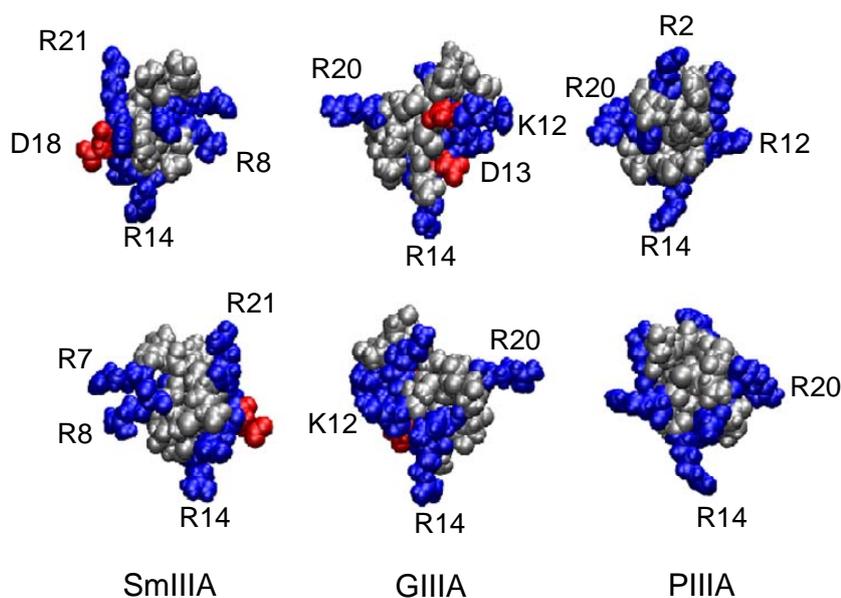
Figure 4. Primary sequences of μ -conotoxins from five different species of *Conus*. The consensus numbering defined here is used in the following figure. Blue (positive), red (negative), green (cysteine), pE (pyroglutamate), O (hydroxyproline). Carboxy termini are amidated.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
GIIIA		R	D	C	C	T	O	O	K	K	C	K	D	R	Q	C	K	O	Q	R	-	C	C	A
GIIIB		R	D	C	C	T	O	O	R	K	C	K	D	R	R	C	K	O	M	K	-	C	C	A
GIIIC		R	D	C	C	T	O	O	R	K	C	K	D	R	R	C	K	O	L	K	-	C	C	A
PIIIA	pE	R	L	C	C	G	F	O	K	S	C	R	S	R	Q	C	K	O	H	R	-	C	C	
SmIIIA	pE	R	C	C	N	G	R	R	G	C	S	S	R	W	C	R	D	H	S	R	C	C		
SIIIA	pE	N	C	C	N	G	-	-	G	C	S	S	K	W	C	R	D	H	A	R	C	C		
KIIIA			C	C	N	-	-	-	-	C	S	S	K	W	C	R	D	H	S	R	C	C		

Table 2. Some characteristics of known μ -conotoxins.

μ -Conotoxins	<i>Conus</i> species	# of residues	Nominal net charge (using R, K=1, H=+0.5, D=-1)	Major tissue or channel subtype(s) targeted	References
GIIIA	<i>C. geographus</i>	22	6	Rat muscle	[98]
GIIIB	<i>C. geographus</i>	22	7	Rat muscle, rat brain	[98]
GIIIC	<i>C. geographus</i>	22	7	Rat muscle	[33; 98]
PIIIA	<i>C. purpurascens</i>	22	6.5	Rat muscle, rat brain, human brain	[98; 134]
SmIIIA	<i>C. stercusmuscarum</i>	22	5.5	TTx-r (amphibian) skeletal muscle, sympathetic and dorsal root ganglia (DRG) neurons	[19; 73; 156]
SIIIA	<i>C. striatus</i>	20	2.5	TTx-r (amphibian) sympathetic and DRG neurons	[19]
KIIIA	<i>C. kinoshitai</i>	16	3.5	TTx-r (amphibian) sympathetic and DRG neurons	[19]

Figure 5. NMR-derived structures for three μ -conotoxins. Atomic coordinates were taken from RCSB Protein Data Base [10]; coordinates used were as follows: GIIIA, 1TCG; PIIIA, 1RD1, structure #19; SmIIIA, 1Q2J, structure #19. Software for figure generation was as in Figure 2. The conotoxin molecules are oriented in similar directions with an axis along the R14 side-chain facing down. In the lower set, images are rotated 180 degrees around the R14 axis with respect to the upper set. Positively charged residues are shown in blue; negatively charged residues are shown in red. Residue numbering follows the consensus numbering from the alignment in Figure 4.



4.3.2. μ O-Conotoxins

The μ O- and δ -conotoxins are unusually hydrophobic peptides, which belong to the O-superfamily of conotoxins. The δ -conotoxins inhibit inactivation of Na_v channels, while μ O-conotoxins affect both Na^+ and Ca^{2+} currents [48]. μ O-conotoxins were first isolated from the cone snail, *Conus marmoreus* [48; 95]. *C. marmoreus* is a molluscivorous (snail hunting) cone snail which can be found in the waters of the Philippines [95]. To date, only two μ O-conotoxins have been discovered, the MrVIA and MrVIB, both from *C. marmoreus*. Both of these toxins are 31 residues long, with three intramolecular disulphide bonds. They show high sequence homology with only two residues not conserved between the two toxins. The 3D solution structures of these toxins were first determined using simulated annealing procedures and based on NMR data [34]. The μ O-conotoxins inhibit Na_v channels as well as molluscan calcium channels [48].

The chemical structure of μ O-conotoxins has similarities with δ -conotoxins, and the ω -conotoxins (which target calcium channels and not discussed further in this chapter) [34]. The block of Na_v channels by the μ O-conotoxins seems to be independent of site 1, at which TTx, STx, and the μ -conotoxins bind, and shows no effect on either activation or inactivation of the channel [146]. Competition studies showed that the μ O-conotoxins have no effect on the binding of [^3H]STx. Furthermore, targeting of μ O-conotoxins differs significantly from the μ -conotoxins. As well as

inhibiting TTx-s Na_V channels from brain ($\text{Na}_V1.2$) with IC_{50} (concentration producing 50% inhibition) values of 200 nM [146], they also block TTx-r Na_V channels from the rat dorsal root ganglion (probably $\text{Na}_V1.8$) with IC_{50} values of 82.8 nM. The equivalent TTx-r channels in humans are responsible for transmission of pain sensation, which makes μO -conotoxins a very promising therapeutic agents [34]. However, the molecular mechanisms of interaction of the μO -conotoxins with Na_V and Ca_V channels are not yet well understood.

Table 3. Some characteristics of known μO -conotoxins.

μO -Conotoxins	<i>Conus</i> species	Number of residues	Subtype Targeted	Reference
MrVIA	<i>C. marmoreus</i>	31	Inhibits TTx-r Na_V channels rat dorsal root ganglia, TTx-s Na_V channels, molluscan Ca^{2+} channels	[34; 95]
MrVIB	<i>C. marmoreus</i>	31	Inhibits TTx-r Na_V channels rat dorsal root ganglia, TTx-s Na_V channels, molluscan Ca^{2+} channels	[34; 95]

4.3.3. Unclassified conotoxins, GS and TVIIA

The unusual properties of some conotoxins do not allow them to be grouped within the known families [144]. For example, TVIIA from the venom of *Conus tulipa*, a 30-residue polypeptide, displays high sequence identity with conotoxin GS, from venom of *Conus geographus*. Although there are reports indicating that GS binds to the TTx receptor in rat skeletal muscle, no more detailed functional studies have appeared. Their overall structure suggests that GS and TVIIA represent a distinct subgroup of the family which includes the Ca_V -targeted ω -conotoxins [62].

Table 4. Two unclassified conotoxins.

Unclassified Conotoxins	<i>Conus</i> species	Number of residues	Subtype Targeted	Reference
TVIIA	<i>C. tulipa</i>	30	Not established	[62]
GS	<i>C. geographus</i>	34	Muscle Na^+ blocker receptor site 1	[163]

4.4. Lipopeptide Na_V channel inhibitors

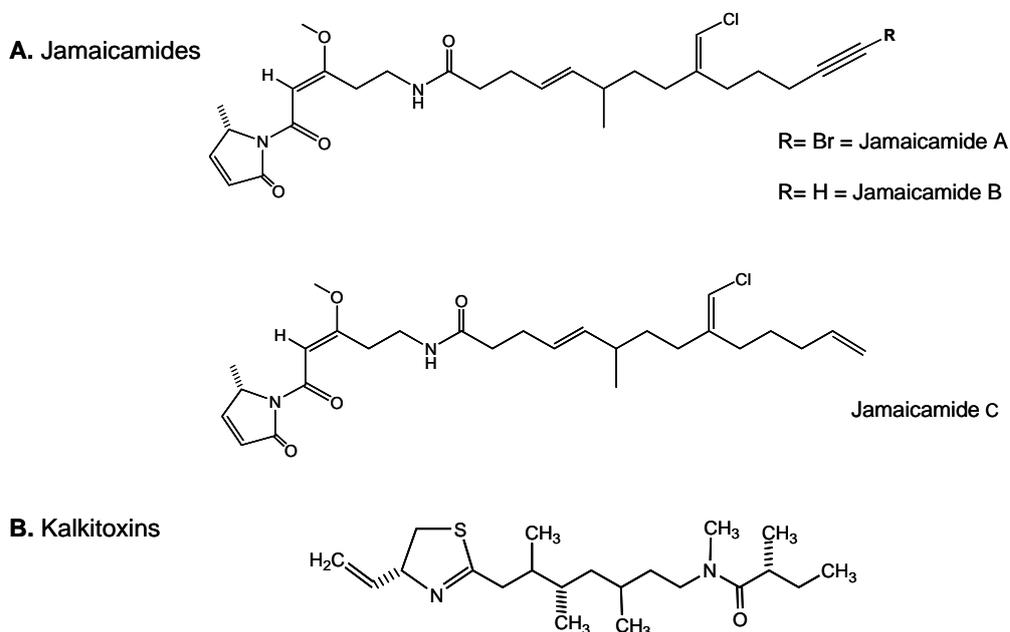
4.4.1. Jamaicamides

Screening extracts from marine Dinoflagellate *Lyngbya majuscula* revealed a number of potentially bioactive metabolites ([20] and reviews therein). Jamaicamides A-C (Figure 6-A) are recently characterized polyketide-peptide neurotoxins from *L. majuscula* [38]. The latter authors examined the neurotoxic activity of crude extracts as well as purified jamaicamide compounds. Neither jamaicamide

A nor B showed significant brine shrimp toxicity, while jamaicamide C was only modestly active at 10 ppm (25% lethality).

In the goldfish toxicity assay, jamaicamide B was the most active (100% lethality at 5 ppm after 90 min), followed by jamaicamide C (100% lethality at 10 ppm after 90 min). Interestingly, jamaicamide A was the least active fish toxin (sublethal toxicity at 10 ppm after 90 min). Also, Jamaicamide compounds exhibited cytotoxicity to both the H-460 human lung and neuro-2a mouse neuroblastoma cell lines. Using a bioassay method, modified from Manger et al. [91], it has been suggested Na_V channel modulation by jamaicamides A-C [38]. The LD_{50} s from these bioassays were approximately 15 μM for all three compounds in both cell lines. All three compounds also inhibited Na_V channel activity at 5 μM , producing approximately half the response of STx applied at 0.15 μM . None of the jamaicamides were shown to activate the tested Na_V channels. Overall, jamaicamides show a spectrum of biological activities consistent with their functioning as defense metabolites, including Na_V channel blocking activity, and arthropod and fish toxicity. Further work should be done to study the molecular interaction of jamaicamide compounds with Na_V channels.

Figure 6. Chemical structures of some Na_V channel peptide blockers. **A.** Jamaicamides A-C: lipopeptides from dinoflagellate *Lyngbya majuscula*. **B.** Kalkitoxin: a thiazoline-containing lipopeptide toxin also from dinoflagellate *L. majuscula*. The structures of jamaicamides were reproduced from Edwards et al.[38], with permission. The structure of kalkitoxin was reproduced from LePage et al.[81], with permission.



4.4.2. Kalkitoxin

Kalkitoxin (KTx) is a neurotoxic, thiazoline-containing lipopeptide (see Figure 6-B) from a Caribbean dinoflagellate, *Lyngbya majuscula* [160]. This toxin acts exclusively as a potent blocker of activated TTx-s Na_V channels in mouse neuron-2a cells (50% block occurred at $\text{IC}_{50} = 1 \text{ nM}$, compared to the STx $\text{IC}_{50} = 8 \text{ nM}$; [160]). In another study, KTx produced *N*-methyl-D-aspartate (NMDA) receptor-dependent cytotoxicity in rat cerebellar granule neurons, with an LD_{50} of $3.86 \pm 1.91 \text{ nM}$ [9]. It is somewhat surprising that the agonist, antillatoxin (ATx, see below), also induced

cytotoxicity that was prevented by non-competitive NMDA receptor antagonists. Nevertheless, the cytotoxicity responses to ATx and KTx displayed distinct temporal patterns [9], in that ATx induced an acute response, whereas KTx produced delayed cytotoxicity. Thus, cytotoxicity may be induced by different pathways in these two cases. Additionally, KTx is highly active in an inflammatory disease model which measures IL-1 β -induced secreted phospholipase A₂ secretion from HepG2 cells (IC₅₀ = 27 nM; [160]). Recently, KTx was shown to interact with TTx-s Na_v channels at site 7 in cerebellar granule neuron cultures [81]. Consistent with previous work, KTx may be a valuable tool in efforts to more clearly define the structure-function relationships of these Na_v channels, and may provide possible leads for analgesic and neuroprotection drug discovery.

5. Gating modifiers

5.1. Non-peptide Na_v channel modulators

5.1.1. Alkaloid inhibitors of Na⁺ channels

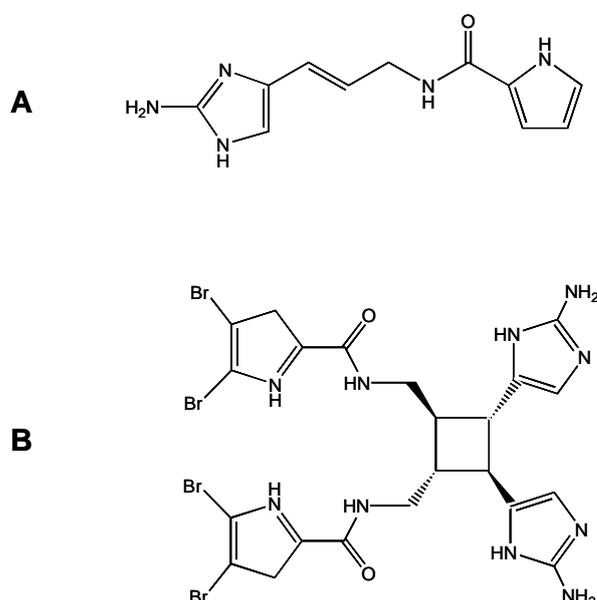
Marine organisms are known to produce compounds with novel chemical structures and interesting biological activities. Apart from algae, cone snails, sea anemones and many other organisms, sponges are the source of the greatest diversity of marine natural products (see references [15; 50] and previous reviews cited therein). Alkaloid compounds found in tropical *Agelas* sponges have been shown to be active on muscle and nerve membrane receptors and channels, including Na_v channels, and are excellent pharmacological tools to study the physiology and biophysics of these macromolecules [119]. Morales and Rodriguez [96] reported that Caribbean sponges of the genus *Agelas* contain alkaloids including monomers of clathrodin (CLA, Figure 7-A) and dibromosceptrin (DBS, Figure 7-B). Both compounds have guanidine moieties linked to pyrrol groups with bromide substitutions ranging from none (CLA) to four (DBS). Use of the whole-cell configuration of the patch-clamp technique revealed that these compounds have neurotoxic activity [119]. Both compounds decreased the average maximum amplitude of inward Na⁺ current by 27-40% on cells isolated from sympathetic ganglia of chick embryo to test the CLA and DBS effect on provoked Na⁺ currents. In this study, neither compound altered the voltage dependence of current activation.

However, the voltage dependence of current inactivation was shifted toward more negative potentials by DBS. In contrast, CLA shifted this voltage dependence of inactivation toward more positive potentials. Time for current reactivation was not altered by CLA but was slightly prolonged by DBS. Similarly, DBS was more effective than CLA in prolonging the time course of current decay. Voltage clamp experiments on frog muscle showed that both CLA and DBS alter the voltage-dependent ionic currents, with CLA causing an early potentiation followed by total inhibition, and DBS exerting an immediate current blockade [119].

CLA and DBS effects on ionic channels were examined using CLA and DBS in radioligand binding studies, which showed that DBS inhibited nearly 90% of [³H]-STx binding to rat brain synaptosomes while CLA inhibited only 30% [119]. Thus, CLA and DBS are Na_v channel neurotoxins which bind to a site that inhibits STx binding. Nevertheless, further investigation is needed to specify the molecular target of each toxin. Functionally, CLA and DBS modulate Na_v channels by different mechanisms; DBS, a dimer with four bromide substitutions, acted on the voltage-dependence of

inactivation, while CLA, the monomer without bromide substitutions, was directed towards the channel ionic conductance. Recently, DBS has been shown to disrupt the cellular calcium homeostasis, by targeting Ca_v channels ($\text{IC}_{50} \sim \mu\text{M}$) in PC12 cells [11]. This potent effect of DBS on Ca^{2+} influx and Ca^{2+} levels in neurons and sensory cells is consistent with that the previously reported feeding deterrent activity of brominated pyrrole alkaloids in *Agelas* sponges against predatory reef fish (reviewed by [87]). Furthermore, chemoreception, sensing bromopyrrole alkaloids in sea water, was exhibited by sensory neurons in the rhinophore of the sea slug *Aplysia punctata* [11].

Figure 7. Chemical structures of non-peptide gating modifiers of Na_v channels. **A.** Clathroodin (CLA) and **B.** Dibromosceptrin (DBS) alkaloid toxins from *Agelas* sponge. The chemical structure of CLA was reproduced from Morales and Rodriguez [96], with permission; the structure of DBS was reproduced from Bickmeyer et al [11], with permission.



5.1.2. Brevetoxins

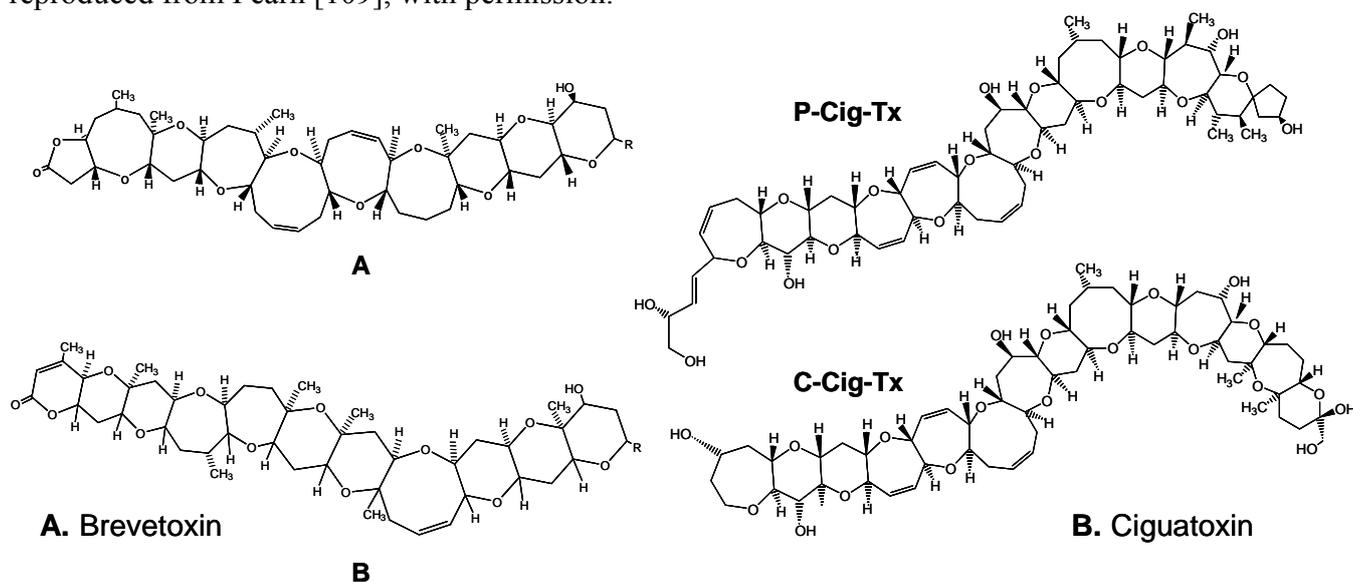
The brevetoxins, are part of a large family of dinoflagellate-derived polyketide toxins which pose a threat to human health through the consumption of tainted seafood. This family also includes ciguatoxin, okadaic acid and the related dinophysins, pectenotoxins, yessotoxin and the azaspir acids [136]. Ingestion of bivalve molluscs contaminated with brevetoxins leads to neurotoxic shellfish poisoning (NSP), the symptoms of which include nausea, cramps, paresthesias of lips, face and extremities, weakness and difficulty in movement, paralysis, seizures and coma [4; 39; 94]. Inhalation of aerosolized brevetoxins from sea spray results mainly in respiratory irritation as well as dizziness, tunnel vision and skin rashes [4; 112].

Brevetoxins (PbTx-1 to PbTx-10) are potent lipid-soluble polyether neurotoxins produced by the marine dinoflagellate, *Karenia brevis* (formerly known as *Gymnodium breve* or *Ptychodiscus breve*; [2]). These organisms are associated with 'red tide' blooms in the Gulf of Mexico and in New Zealand [69]. Brevetoxins exert a variety of actions *in vivo* affecting both the PNS as well as the CNS, probably due to their high lipid solubility and ability to penetrate the blood brain barrier [78]. The brevetoxins are a suite of polyether ladder type compounds which have two parent backbone

structures, brevetoxin A and brevetoxin B, each with several side chain variants [3] (Figure 8-A). Brevetoxin 1, PbTx-1 from group A, is the most active ichthyotoxic brevetoxin analog with IC₅₀ values ranging between 3 and 4 nM [2].

Brevetoxins are known to interact specifically with neurotoxin receptor site 5 on the α subunit of Na_v channels [2]. This interaction with the α subunit causes a shift in the voltage dependence of channel activation to more negative potentials and inhibits channel inactivation, thereby producing neuronal depolarization at rest [26; 113; 116; 117; 129]. In addition, brevetoxins augment Na⁺ influx through Na_v channels because of the shift in activation, and by prolonging channel opening, resulting in a dose-dependent depolarization of excitable membranes [72]. Bottein Dechraoui and Ramsdell [16] showed that type A (PbTx-1) and type B (PbTx-3 and PbTx-2) brevetoxins, as well as ciguatoxins, target both cardiac and muscle channels. Type B brevetoxins show isoform selectivity, presenting a lower affinity for the cardiac (Na_v1.5) than the skeletal muscle (Na_v1.4) Na_v channel isoforms expressed in Human Embryonic Kidney (HEK) cells. The lower affinity of type B brevetoxins for heart Na_v channels may result from a more rigid backbone structure than that found in type A brevetoxins and in ciguatoxins. On the other hand, PbTx-2 augmented NMDA receptor function and, therefore, may exacerbate NMDA receptor-mediated pathologic sequelae associated with neurodegenerative diseases [36]. Furthermore, a series of naturally occurring fused-ring polyether compounds with fewer rings than brevetoxin, known as brevenals, were shown to exhibit antagonistic properties and counteract the effects of the brevetoxins in neuronal and pulmonary model systems [3]. This finding supports the idea that the toxicity of the red tide blooms depends on the amount of each toxin present as well as the spectrum of molecular activities elicited by each toxin.

Figure 8. Molecular structures of polyether ladder toxins which are Na_v channel modifiers. **A.** Brevetoxins A and B are isolated from marine dinoflagellate, *Karenia brevis*. **B.** Ciguatoxins (Pacific; P-CigTx and Caribbean; C-CigTx) from marine dinoflagellate, *Gambierdiscus toxicus*. Structures of brevetoxins were reproduced from Baden et al. [3], with permission. Structures of ciguatoxins were reproduced from Pearn [109], with permission.



5.1.3. Ciguatoxins

The term ciguatera originated in the Caribbean areas to designate intoxication induced by the ingestion of the marine snail *Turbo pica* (called cigua), first described by Cuban ichthyologists (reviewed by [109]). Today, the term is widely used to denote a fish-poisoning that result from ingestion of ciguatoxic fish found mainly in tropical and subtropical regions [83]. Human victims are the end link in a food chain cascade where ciguatoxins are accumulated in a variety of consumed coral reef fishes [82]. Ciguatoxins (CigTx) are lipid-soluble, heat-stable, highly oxygenated cyclic polyether marine toxins originally produced in coral reef areas by the benthic dinoflagellate *Gambierdiscus toxicus*. Ciguatoxins are responsible for a complex human food poisoning, characterized by gastrointestinal, neuronal and cardiovascular disturbances with no immunity and symptoms may persist for months or years [79]. The symptoms of ciguatera fish poisoning overlap those of the brevetoxins with temperature reversal as supposedly unique for ciguatoxins [112]. Moreover, epidemiology of the CigTx poisoning is complex and impossible to predict outbreaks with global health concern. Worldwide coral bleaching, together with man-made factors (such as tourism, industrial constructions, sewage and eutrophication) and environmental factors (such as earthquakes and hurricanes) create a more favorable environment for *G. toxicus* blooms and therefore trigger ciguatera outbreaks [79].

More than 20 CigTx analogs have been found, and grouped according to their origins and carbon backbone, as Pacific (P-CigTx; Figure 8-B) or Caribbean (C-CigTx; Figure 8-B) ciguatoxins [93; 109]. These polyether toxins are highly toxic to fish (LD_{50} 1 $\mu\text{g}/\text{kg}$; [79]) and to mammals (LD_{50} for mouse near 1 $\mu\text{g}/\text{kg}$; [83]). Interestingly, the more oxidized form, P-CigTx, is up to 10-fold more toxic than the less oxidized form, C-CigTx [83; 164]. This increase in potency amplifies the effective level of more oxidized forms of CigTx accumulated by fish. Extensive experimental studies of Pacific ciguatoxins, using whole-cell patch clamp of rat dissociated dorsal root ganglion neurons, have shown that P-CigTx-1 causes TTx-s Na_v channels to open at more negative membrane potentials than normal. By contrast, TTx-r Na_v channels recover from inactivation more quickly, enabling an earlier transition to the open state [138]. These properties, combined with the high lipid solubility of the toxin explain, at least in part, the pathogenic nature of the neurological symptoms, including the pathogenesis of sensory neurological disturbances associated with ciguatoxic fish poisoning.

Pharmacological studies have revealed that ciguatoxins activate Na_v channels in the nM to pM range [7; 12; 16; 83; 89]. Specifically, ciguatoxins cause a hyperpolarizing shift in the voltage-dependence of the channel activation, increasing the open probability of Na_v channels at normal resting membrane potentials [7; 12; 89]. These toxic effects lead to a TTx-s, Na^+ -dependent membrane depolarization, and repetitive or spontaneous action potentials, in various axons and skeletal muscle fibers [16; 83; 93]. Ciguatoxins in common with brevetoxins selectively target a common receptor site, receptor site 5, on Na_v channel proteins [12; 89; 93]. Because of their potency, ciguatoxins can be useful pharmacological tools to study Na_v channels, as are TTx, STx and PbTx. Moreover, an increase of Na^+ influx through activation of these channels at the resting membrane potential, is not only able to enhance nerve excitability but also may cause water movements across the cell membrane [93]. These toxins may be useful tools to study the Na^+ -dependent mechanisms of volume changes and their homeostasis in nerve preparations.

5.1.4. Ostreotoxin-3

More than 20 species of marine dinoflagellates produce toxins that are among the most potent, non-pertinacious toxin known [20; 49; 159; 164]. These dinoflagellate toxins have been implicated in human intoxications resulting from consumption of poisonous fish and shellfish [159; 164]. The benthic dinoflagellate, *Ostreopsis lenticularis*, is the producer of this Na_V channel activating toxin, Ostreotoxin-3 [OTx-3; [44]]. Toxicity of OTx-3 was first revealed when *O. lenticularis* cells treated with acetone before methanol extraction showed a 10-fold increase in mouse toxicity [44]. Experiments in chick embryo sympathetic neurons demonstrated that OTx-3 extracts were acting on TTx-s Na_V currents [44]. The extract affected the voltage-dependence by shifting the activation and inactivation currents to more negative potentials but its major effect was on inactivation. In the presence of μ -conotoxin GIIIA, the remaining Na^+ current was not altered by OTx-3 extracts. Meunier et al. [44] performed intracellular recording experiments which demonstrated that OTx-3-enriched extracts depolarized frog skeletal muscle and decreased the amplitude of endplate potentials without affecting the nerve impulse. This effect was partially blocked by 10 μM TTx and totally blocked by μ -conotoxin GIIIA. Thus, the toxic effect of *O. lenticularis* extracts may be due to the presence OTx-3 that recognized TTx-s Na_V currents. OTx-3 is a unique dinoflagellate toxin that can discriminate between nerve and skeletal muscle.

5.2. Peptide Na_V channel gating modifiers

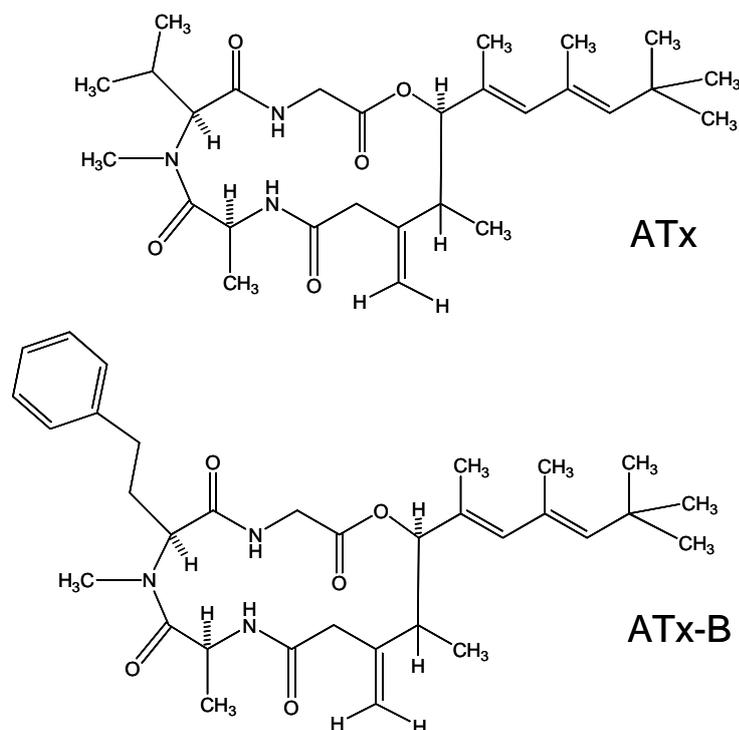
5.2.1. Antillatoxin

Blooms of the dinoflagellate, *Lyngbya majuscula*, have been associated with various adverse effects on human health, including respiratory irritation, eye inflammation, and severe contact dermatitis in exposed individuals [21; 97]. These effects are associated with the production of toxic substances by *L. majuscula* such as lyngbyatoxins [21; 131; 164] and debromoaplysiatoxin [97; 131]. Other toxic secondary metabolites target Na_V channels, (see Table 1); these include antillatoxin [107], kalkitoxin [9; 160] and jamaicamides A-C [38]. The diversity of these natural products gives them potential for importance as biochemical and pharmacological tools.

Antillatoxin (ATx) is a structurally unusual cyclic lipopeptide (Figure 9), which has been reported to be among the most ichthyotoxic metabolite isolated to date from a marine dinoflagellate. It is exceeded in potency only by the brevetoxins [107]. ATx ichthyotoxic activity with LD_{50} value of 0.05 $\mu\text{g}/\text{ml}$ using fish toxicity bioassay [107]. Using cerebellar granule cells, ATx produced concentration-dependent cytotoxicity with LD_{50} value of 20.1 ± 6.4 nM [9]. In the latter study, the neurotoxic response of ATx was prevented by co-application with noncompetitive antagonists of the NMDA receptor such as MK-801 (or dizocilpine) and dextrorphan. This neurotoxic effect of ATx was antagonized by TTx, consistent with an interaction of ATx with Na_V channels [86]. ATx-induced stimulation of Ca^{2+} influx in cerebellar granule cells was similarly prevented by TTx. Additional, more direct, evidence that the Na_V channel serves as the molecular target for ATx was provided by the demonstration that ATx stimulation of [^3H]batrachotoxin binding increases $^{22}\text{Na}^+$ influx in cerebellar granule cells [86]. Together, these results suggest that ATx is a novel activator of Na_V channels, most probably acting at site 4. The precise recognition site for ATx on the Na_V channel, however, remains

to be defined. It has been suggested that development of antagonists targeted at the ATx lipopeptide site may provide another means of inhibiting Na_v channels, and thereby a new strategy for the discovery of anticonvulsant and neuroprotectant drugs [86].

Figure 9. The cyclic lipopeptide structures of antillatoxins, ATx and ATx-B, from *Lyngbya majuscula*. The structures of antillatoxins were reproduced from Li et al. [86], with permission.



Recently, a new *N*-methyl homophenylalanine analog of ATx was identified and denoted as antillatoxin B (ATx-B; [101], Figure 9). Both ATx and ATx-B are potent activators of the Na_v channel in mouse neuro-2a neuroblastoma cells, with half-maximal activation at EC_{50} values of 0.18 and 1.77 μM , respectively. In addition, ATx B is strongly ichthyotoxic to goldfish, with an LD_{50} of 1.0 μM . It seems likely that substitution of a larger *N*-methyl homophenylalanine residue in ATx-B for an *N*-methyl valine residue in ATx accounts for the 10-fold decrease in ATx-B's potency in compare to ATx [101].

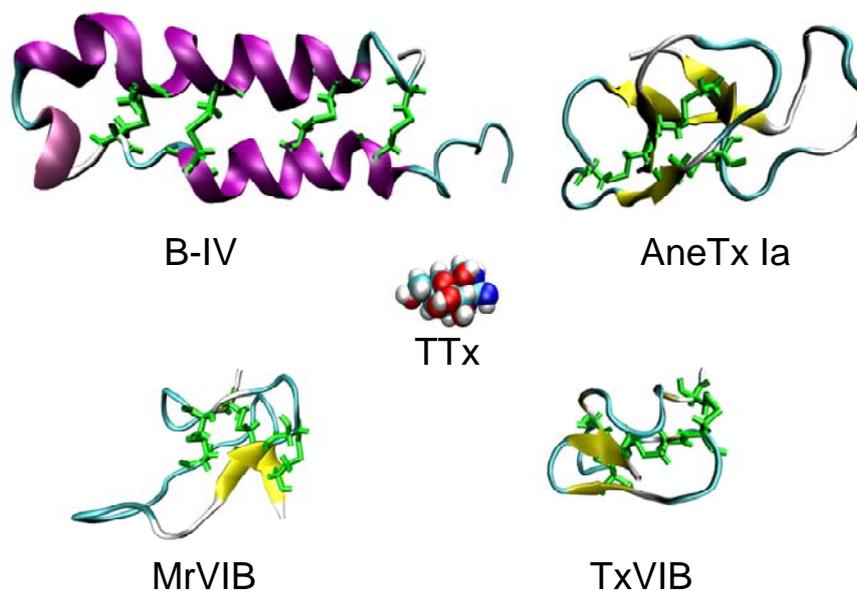
5.3. Peptide gating modifiers

5.3.1. Sea anemone toxins

Sea anemones are a member of the phylum Cnidaria, which have stinging organelles known as nematocysts, housing tiny harpoons, on their tentacles. Sea anemones have adapted their venom apparatus for use in both protection and prey capture. They have been known to capture both small fish and crustaceans. Toxins produced by sea anemones encompass many targets including both K_v and Na_v channels, while some species have cytolytins. Sea anemones have a wide variety of Na_v channel toxins that affect inactivation from the extracellular side of the channel. Sea anemone toxins

were first isolated as cardiac stimulators and neurotoxins and these are the two actions that have been studied the most. Sea anemone toxins affecting Na_v channels range in size from 3-5 kDa and have been isolated from many different species [102]. Sea anemone toxins bind to Na_v channels of excitable tissue and preferentially act on the open state to inhibit inactivation of the channel [35]. Sea anemone toxins bind to site 3 on the Na_v channel [27] in domain IV on the S3-S4 linker. The binding to this linker prevents the necessary movement of the voltage sensing S4 segment during fast inactivation. Sea anemone toxins bind to a receptor which partially overlaps that of scorpion toxins, as seen in binding studies of the interaction between the sea anemone toxin, AneTx II, and the scorpion toxin, LqTx [121].

Figure 10. Examples of peptide toxin backbone structures shown with disulphide bonds (green) created with VMD [68] of B-IV [6], AneTx Ia [158], μO -conotoxin MrVIB [34] and δ -conotoxin TxVIB [77].



Sea anemone toxins, because of their large variety, can be split into three distinct groups called Type-1, Type-2 and Type-3. These groups are distinguished based on both their size and the genus from which they were isolated. Type-1 and Type-2 sea anemone toxins usually range from about 46-49 amino acids in length. Type-1 toxins were isolated from the family *Actiniidae*, mostly from the genera *Anemonia* and *Anthopleura*. Both of these genera produce several neurotoxins that affect Na_v channels. For example, AFT I, AFT II [141], Anthopleurin-A [142], Anthopleurin-B [115], Anthopleurin-Q [53], and APE 1 to APE 5 [17], are produced by *Anthopleura* spp., while AneTx I, AneTx II, AneTx V [125; 161; 162] are produced by members of the genus *Anemonia*. Type-2 toxins have been isolated from two genera *Heteractis* and *Stichodactyla*. Examples of type-2 toxins are RTX I to RTX V [168-171], Rp II [152], Rp III [44] and ShI [75]. Type-1 toxins, like Type-2 toxins, have 3 disulphide bonds [102]. Within each type, these two toxin subclasses show high sequence identity (>60%), but they show only about 30% identity between Type-1 and Type-2 toxins. Alignment of Type-1 and Type-2 toxins reveals a conserved Arg-14. It is believed that the loop of residues

surrounding the Arg-14 is important in the binding of these toxins to the channel [127]. The C-terminus is more basic in the Type-2 toxins based on the addition of several lysines and a compensating truncation of the N-terminus [102]. In all, of the Type-1 and Type-2 toxins for which structures have been determined to date, all contain a four-stranded anti-parallel β -sheet in their secondary structure, with no apparent helical structure, as shown in Figure 10. Competition studies of these two types of toxins suggest that they bind to distinct yet close receptor sites, given that some toxin pairs show competition between the two groups, while others do not [102].

Type-3 toxins comprise a small group of short polypeptides produced by sea anemones. Some examples of Type-3 toxins are PaTx from *Parasicyonis actinostoloides* [99], AneTx III from *Anemonia sulcata* [8], as well as three others from *Dofleinia armata* (Da I and Da II) and *Entacmaea ramsayi* (Er I) [66]. Type-3 toxins are usually composed of 27-31 residues, all so far with high sequence homology, with AneTx III and PaTx showing the lowest amino acid identity of approximately 50% [66]. Similar to Type-1 and Type-2 toxins, Type-3 toxins seem to bind to a receptor which overlaps position on site 3, and cause a slowing of the inactivation of Na_v channels.

5.3.2. Goniopora Toxin

Goniopora toxin (GPT) was first isolated from the marine coral, *Goniopora spp.* GPT is a polypeptide toxin of approximately 12,000 Da [60]. Electrophysiological experiments have shown GPT to prolong the action potential in heart, without significantly affecting its amplitude, or the resting membrane potential [54]. GPT inhibited inactivation of the cardiac Na_v channels, thus causing an increased permeability to Na^+ . The resultant broadening of the action potential leads to cardiac arrhythmias. These physiological effects are similar to those of several other Na_v channel toxins, including sea anemone toxins and α -scorpion toxins, even though there is little or no sequence homology among them [60].

In N18 neuroblastoma cells, GPT shifts the conductance-voltage relationship to the left, and thus causes activation of sodium currents in response to an unusually small depolarizing stimulus [58]. This result is similar to that produced by the *Leiurus* scorpion toxin. GPT also shifts the steady state inactivation curve of the Na_v channels to the right without altering its shape, in contrast to sea anemone and scorpion toxins, which cause a reduction in the steepness of the inactivation curve while not shifting the midpoint appreciably [57]. Depolarization has a marked effect on the affinity of GPT. The voltage dependence of GPT action reflects an e-fold increase in K_d per 48.3 mV of depolarization [57]. Again, this result differs from that observed for sea anemone toxins, which shows much steeper voltage dependence. Thus, GPT may bind preferentially with different voltage-sensitive states, or alter different voltage-sensitive transitions of the channel.

GPT shows no competitive binding with the scorpion toxins which bind to site 3. Thus, it has been determined that GPT must bind to a distinct site (site 8) to that of the scorpion toxins, even though they both preferentially slow channel inactivation.

5.3.3. B-toxins

The B-toxins were isolated from the mucus secreted by the marine heteronemertine *Cerebratulus lacteus* [74]. *C. lacteus* is a ribbon worm found off the Atlantic coast of North America. B-toxins bind to Na_v channels in crustaceans, but had no effect when they were injected into an insect (cockroach) or a mammal (mouse) [74]. They appear to be selective for crustacean neuronal action potentials. The B-toxins, with molecular masses of ~6,000 Da, are a group of neurotoxins comprised of 4 subtypes, B-I to B-IV of which the B-I toxin (49 residues) contains three intrachain disulfide bonds, while B-II, B-III and B-IV (53 or 54 residues) contain four intrachain disulphide bonds [74]. These B-toxins act by delaying the inactivation of neuronal Na_v channels, as do the α -scorpion and sea anemone toxins that act at site 3 [27]. There is little sequence similarity between B-toxins and the α -scorpion and sea anemone toxins [13]. The B-toxin is composed of two anti-parallel α -helices cross-linked by disulphide bonds forming a hairpin structure, while α -scorpion and sea anemone toxins are mainly β secondary structures [6]. Of the four B-toxins, B-II shows the highest toxicity and is some 15-fold higher than that of B-IV, despite their high overall sequence similarity [74].

To date, the best studied of the B-toxins is B-IV, which is the most abundant form. The B-IV toxin was first sequenced by Blumenthal and Kem in 1976 [14] and it is reported to be the first marine invertebrate polypeptide neurotoxin sequenced. The NMR solution structure of the B-IV toxin has been solved by Norton and colleagues [6] and is shown in Figure 10. There are many charged residues, which could play a role in interacting with the Na_v channel α subunit. Site-directed mutagenesis of the B-IV toxin has shown that neutral replacement of its arginine residues can almost abolish its biological activity [153]. Specifically, replacement of Arg-17 and Arg-25 [67], in the N-terminal helix and the hairpin region respectively, led to at least a 700-fold loss in activity. Furthermore, replacement of Trp-30 with a non-aromatic residue caused a greater than 40-fold decrease in toxicity, whereas aromatic substitutions caused less than 5-fold decrease in toxicity [154].

5.3.4. δ -Conotoxins

Cone snail δ -conotoxins [144], are a group of peptides consisting of about 30 amino acid residues, which exert a similar effect on Na_v channels. This effect is the inhibition of the fast inactivation of Na⁺ currents, which causes a marked prolongation of action potentials [47; 61]. This results in a hyperexcited state of the affected cells, which can eventually lead to a massive electrical hyperexcitation in the complete organism. δ -Conotoxins belong to the O-superfamily and have the same cysteine framework as the μ O- and ω -conotoxins, that target Na_v and Ca_v channels, respectively [144]. This disulfide bonding pattern gives these peptides an inhibitory cysteine knot motif which is found in a variety of biological active peptides from different organisms [103].

Olivera and collaborators have coined the term “cabal” for a group of toxins, present in a venom, which acts together toward a specific physiological end. The set of venom peptides that almost instantaneously immobilize a fish by eliciting the seizure-like symptoms is referred to as the “lightning-strike cabal” [105]. δ -Conotoxins, which in effect cause a dramatic increase in electrical activity of axons, may well be a key venom component in the lightning-strike cabal [132; 145].

It has not yet been established how widely distributed δ -conotoxins are among cone snails, especially fish-hunting cones. Every Indo-Pacific fish-hunting cone snail species examined, that is

known to use a “harpoon-and-line” strategy for prey capture, expressed δ -conotoxin in its venom duct [18]. Several δ -conotoxins from fish- and snail-hunting *Conus* species have been identified and the biological importance of these peptides for the capture of prey seems evident. Similar to other conotoxin families which interact with Na_V channels, δ -conotoxins target specific isoforms of Na_V channels with major differences among vertebrate and invertebrate systems (Table 5). δ -PVIA is a 29 amino acid peptide, from the fish-hunting cone snail *C. purpurascens*, which slows fast inactivation of $\text{Na}_V1.2$ -mediated currents expressed in the *Xenopus* expression system as well as of Na^+ currents recorded from hippocampal neurons in culture [145]. More recently, it has been shown that the peptide also affects $\text{Na}_V1.4$ - and $\text{Na}_V1.6$ -mediated currents [124]. By using rat brain synaptosomes, it was demonstrated that δ -PVIA competes with δ -TxVIA for the same receptor site, even though δ -TxVIA is non-toxic in vertebrate systems. When δ -PVIA is administered to fish, it causes contraction of specific muscles resulting in a characteristic extension of the mouth, which was termed “lockjaw syndrome” [132]. Although δ -PVIA elicits excitatory symptoms in both mice and fish, this peptide is inactive in molluscs, even at doses 100-fold higher. During the envenomation of a prey fish, the excitatory effects of δ -PVIA act synergistically with the K_V channel-blocking peptide κ -PVIIA, resulting in the almost immediate, titanic paralysis of the prey [145].

δ -TxVIA from *C. textile* was originally called the “King Kong” peptide because it causes a characteristic dominant behaviour in lobster upon injection [65]. This peptide prolongs inactivation of Na^+ currents only in molluscan neuronal membranes, whereas in vertebrates, δ -TxVIA binds to Na_V channels but without any toxic effects *in vivo* and *in vitro* [45; 46]. Thus, δ -TxVIA can be used as molecular probe to discriminate between molluscan and mammalian Na_V subtypes on the basis of structural differences and activity but not binding [130]. δ -NgVIA, isolated from piscivorous snail *C. nigropunctatus*, competes with δ -TxVIA for binding to rat brain synaptosomes and molluscan central nervous system, and strongly inhibits Na^+ current inactivation in snail neurons, as does TxVIA [47].

δ -GmVIA, isolated from *C. gloriamaris*, causes action potential broadening in *Aplysia* neurons [133]. This peptide also affects $\text{Na}_V1.2$ and $\text{Na}_V1.4$ at micromolar concentrations but does not affect $\text{Na}_V1.6$ -mediated Na^+ currents. A 26-residue peptide (Am 2766) has been isolated and purified from the venom of the molluscivorous snail, *Conus amadis* [140]. Conotoxin Am 2766, reported by the same group, belongs to the δ -conotoxin family and inhibits the decay of the Na^+ current in brain $\text{rNa}_V1.2$ channels, stably expressed in Chinese Hamster Ovary (CHO) cells. The δ -conotoxin family member from *C. striatus*, δ -SVIE inhibited fast inactivation of Na_V channels in dissociated frog sympathetic neurons, and prolonged action potentials on the frog neuromuscular junction [18]. In the same work, δ -SVIE was a potent excitotoxin, when injected into mice, and competed with δ -TxVIA and δ -PVIA for binding to rat brain membranes in autoradiographic experiments. δ -SVIE proved to be highly potent against $\text{rNa}_V1.4$ channels expressed in mammalian cells with a K_d value in the nanomolar range [80]. Both δ -SVIE and δ -PVIA conotoxins slowed the time-course of inactivation of Na^+ currents and shifted the voltage-dependence of activation and steady-state inactivation to more hyperpolarized potentials in frog sympathetic neurons [157].

δ -EVIA, a 32-residue polypeptide from *C. ermineus*, is the first conotoxin known to inhibit Na_V channel inactivation in neuronal membranes from amphibian and mammals (subtypes $\text{rNa}_V1.2$, $\text{rNa}_V1.3$ and $\text{rNa}_V1.6$) without affecting rat skeletal muscle (subtype $\text{rNa}_V1.4$) and human cardiac muscle (subtype $\text{hNa}_V1.5$) Na_V channels [5]. The NMR structures of δ -EVIA and δ -TxVIA were

solved by Volpon et al. [150] and Kohno et al. [77], respectively. Of particular interest in the structure of δ -conotoxins, is the relatively high number of conserved hydrophobic residues exposed on the surface of the toxins. There is some evidence that extracellular binding of these peptides is important to affect inactivation of the channels and that the hydrophobicity of these peptides is probably important for their action. Previous competition experiments measuring the binding of radioactively labeled toxins to membrane preparations containing Na_V channels have suggested that their molecular receptor, site 6, is distinct from that of scorpion α -toxins [46; 47; 130]. The molecular target of δ -conotoxins action on Na_V channels was recently identified. Leipold et al [80], showed that δ -conotoxin SVIE interacts with a conserved hydrophobic triad (YFV) in the domain IV voltage sensor of mammalian Na_V channels. These findings support the hypothesis of hydrophobic interaction of δ -conotoxins with Na_V channels [77; 150]. In Na_V channels, the S3-S4 linker in domain IV is known to harbor parts of both receptor sites 3 and 6, where site 3 is located more toward S3 and site 6 toward S4 (see Figure 2). This channel section forms the major part of a “receptor site”, i.e., receptor site 3 for α -scorpion toxins and receptor site 6 for δ -conotoxins. With a second moiety, the toxins attack the DIV voltage sensor, whose movement is tightly coupled to the process of inactivation [80].

Table 5. Representatives of δ -conotoxins and their subtype targets.

δ -Conotoxin	<i>Conus</i> species	Natural prey	No. of residues	Subtype Targeted	Reference
PVIA	<i>C. purpurascens</i>	Fish	29	Vertebrate preparations only (including fish, frog and mammalian Na_V 1.2; Na_V 1.4 and Na_V 1.6).	[132; 157] [124; 145]
EVIA	<i>C. ermineus</i>	Fish	32	Amphibians and mammalian preparations (r Na_V 1.2, r Na_V 1.3 and r Na_V 1.6 but not muscular r Na_V 1.4 and cardiac h Na_V 1.5)	[5; 150]
SVIE	<i>C. striatus</i>	Fish	31	Diverse (Molluscan and Amphibian sympathetic neurons)	[18; 157]
Am2766	<i>C. amadis</i>	Molluscs	26	Diverse [molluscan and mammalian preparations (r Na_V 1.2)].	[140]
TxVIA	<i>C. textile</i>	Molluscs	27	Molluscan subtype and rat brain neuronal membranes but not toxic to vertebrate system	[45-47; 65; 77]
GmVIA	<i>C. gloriamaris</i>	Molluscs	29	Diverse (molluscan subtype and mammalian Na_V 1.2 and Na_V 1.4 but not Na_V 1.6-mediated Na^+ currents).	[61; 133]
NgVIA	<i>C. nigropunctatus</i>	Molluscs	31	Diverse (molluscan and more potent to mammalian preparations)	[47]

Earlier studies have shown that a δ -conotoxin from the venom of a mollusc-hunting cone snail, δ -TxVIA, does not compete for binding with site 3 toxins [46]. Additionally, a much larger peptide from the venom of *Conus striatus*, CsTx, with an activity similar to that of δ -SVIE, only partially competed with the site 3 α -scorpion toxin LqTx [59]. For these reasons, the receptor site for δ -conotoxins has been defined as site 6 [27]. In the same context, bulky δ -conotoxins, such as δ -SVIE bind in the S3–S4 linker in domain IV and may overlap between site 3 and site 6. Likewise, it has been shown for α -scorpion toxins that critical residues for site 3 binding are found in the S3–S4 linker in domain IV [121]. The α -scorpion toxin receptor site suggests a mechanism by which these toxins exert their effects; namely, binding of toxin to site 3 inhibits the movement of the S4 voltage sensor in domain IV

[27; 121]. “Voltage-sensor trapping” appears to be a mechanism employed by a variety of peptide toxins that interact with Na_V channels [25], and these results suggest that δ -conotoxins act in a similar fashion.

5.3.5. *Conus striatus* toxin

Conus striatus toxin (CsTx), a 25,000 Da polypeptide toxin from the marine cone snail *Conus striatus*, was first isolated by Kobayashi, Ohizumi and colleagues [76]. CsTx binds to Na_V channels causing a slowing of Na_V channel inactivation in both muscle and nerves [40; 43] as well as spontaneous activity in the cardiac musculature [41], while showing no effects on the activation of the channel [59]. CsTx induces a shift in the voltage dependence of steady state inactivation to more negative membrane potentials [59], which is opposite the effect of *Goniopora* toxin [57]. CsTx has been shown to have a positive inotropic effect in the heart [42] similar to that of *Goniopora* toxin. The work by Gonoï et al. [59] showed that CsTx appears to bind in a one-to-one fashion with the channel and its action seems to be voltage dependent with an e-fold increase in apparent K_d per 19 mV depolarization. CsTx has a similar mode of action to the δ -conotoxins, although it only partially competed with the site 3 scorpion toxin α -LqTx [59]. This suggests that there may in fact be a partial overlap between the δ -conotoxins and CsTx [47]. Similar to the α -scorpion toxins, CsTx enhances toxin binding to site 2 (e.g., batrachotoxin binding) through an allosteric mechanism, though to a lesser degree than do the α -scorpion toxins [59].

6. Concluding remarks

In this article, we have attempted to outline the remarkable variety of Na_V channel-targeted toxins which occur naturally in the marine environment. In trying to make our coverage reasonably comprehensive, but still keep the article concise, we have often cited more detailed reviews in specific areas, rather than comprehensively citing original references. Regarding the choices made, we ask patience and understanding of the authors and colleagues who have produced the myriad of excellent, painstaking primary studies, which ultimately provided the basis for this chapter.

The marine ecosystem offers a source of chemically and functionally diverse toxins that interact with Na_V channels. These have already offered considerable insight into the structure and function of the channel protein and provide a rich array of potential pharmacological agents for experimental studies, as well as inspiration for future drug design.

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