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**Review**

**Insect-selective spider toxins targeting voltage-gated sodium channels**

Graham M. Nicholson

_Neurotoxin Research Group, Department of Medical and Molecular Biosciences, University of Technology, Sydney_

_PO Box 123, Broadway, NSW, 2007, Australia_

* Corresponding author. Associate Professor Graham Nicholson, PhD

Neurotoxin Research Group
Department of Medical & Molecular Biosciences
University of Technology, Sydney
PO Box 123
Broadway, NSW, 2007
Australia
Tel.: +61-2-9514-2230; fax: +61-2-9514-8206
_E-mail address:_ Graham.Nicholson@uts.edu.au
Abstract

The voltage-gated sodium (Naᵥ) channel is a target for a number of drugs, insecticides and neurotoxins. These bind to at least seven identified neurotoxin binding sites and either block conductance or modulate Naᵥ channel gating. A number of peptide neurotoxins from the venoms of araneomorph and mygalomorph spiders have been isolated and characterized and determined to interact with several of these sites. These all conform to an ‘inhibitor cystine-knot’ motif with structural, but not sequence homology, to a variety of other spider and marine snail toxins. Of these spider toxins several show phyla-specificity and are being considered as lead compounds for the development of biopesticides. Hainantoxin-I appears to target site-1 to block Naᵥ channel conductance. Magi 2 and Tx4(6-1) slow Naᵥ channel inactivation via an interaction with site-3. The δ-palutoxins, and most likely μ-agatoxins and curtatoxins, target site-4. However their action is complex with the μ-agatoxins causing a hyperpolarizing shift in the voltage-dependence of activation, an action analogous to scorpion β-toxins, but with both δ-palutoxins and μ-agatoxins slowing Naᵥ channel inactivation, a site-3-like action. In addition, several other spider neurotoxins, such as δ-atracotoxins, are known to target both insect and vertebrate Naᵥ channels most likely as a result of the conserved structures within domains of voltage-gated ion channels across phyla. These toxins may provide tools to establish the molecular determinants of invertebrate selectivity. These studies are being greatly assisted by the determination of the pharmacophore of these toxins, but without precise identification of their binding site and mode of action their potential in the above areas remains underdeveloped.

Keywords: Spider toxins, hainantoxin, Magi toxins, δ-palutoxins, voltage-gated sodium channel, insecticide
1. Introduction

The Na$_v$ channel mediates the increase in sodium conductance during the rapid depolarization phase of the action potential. Therefore this channel represents a key structural element that controls cellular excitability in biological systems. Not surprisingly it has become the target of a variety of plant and animal toxins to assist them in combating predators or in capturing prey animals. Importantly, it is a well-validated target of a range of organic agrochemical insecticides including DDT, N-alkylamines, dihydropyrazoles, oxadiazines and pyrethroids (Sattelle and Yamamoto, 1988; Soderlund and Bloomquist, 1989; Salgado, 1992; Bloomquist, 1996; Narahashi et al., 1998; Zlotkin, 1999; Narahashi, 2000; Raymond-Delpech et al., 2005). However, the real or perceived development of public health concerns (Le Couteur et al., 1999; Betarbet et al., 2000), insect resistance (Brogdon and McAllister, 1998; Soderlund and Knipple, 2003) and environmental hazards (Carson, 1962) has led to a search for novel biopesticides, particularly insect-specific neurotoxins from plants, fungi, bacteria, sea anemone and arachnid venoms. Many are now being investigated for their possible use as bioinsecticidal agents for the control of phytophagous pests or vectors of new or re-emerging diseases.

Spiders have, during their evolution, developed a complex pre-optimized combinatorial peptide library of neurotoxins, enzymes, antimicrobial and cytolytic peptides in their venom glands to diversify their toxin pool (Sollod et al., 2005). New pharmacologies have been produced by hypermutation of the mature toxin sequence resulting in a rich diversity of neurotoxins with high potency and selectivity for multiple cellular targets. This confers an evolutionary advantage for spiders, enabling them to efficiently paralyze and/or kill a wide variety of prey or predators as rapidly as possible. Given that many spiders rely upon their venom to immobilize or kill their prey it is not surprising that they contain a wide variety of insect-selective toxins that may provide the basis for the development of biopesticides. These neurotoxins often target specific insect prey or more precisely subtle differences in the nervous system of their prey. Venoms are a cocktail comprising of both phyla-selective toxins and toxins with a broad spectrum of toxicity. The diversity and complexity of these cocktails enable the spiders to prey either upon specific insect classes (eg. spiders which target moths or pollinating insects) although most are generalist predators and have a broad invertebrate diet (eg. trapdoors and funnel-web spiders) (Nentwig, 1987). Interestingly, a large number of these phyla-specific toxins target voltage-gated ion channels to rapidly modify ion channel gating and kinetics. Given the range of excitatory and inhibitory activities in the venoms of many species, spiders appear to also use ‘toxin cabals’ for rapid paralysis, analogous to the strategy employed by marine cone snails (Olivera, 1997). These represent suitable targets for the future development of insecticides since they are ubiquitous amongst insects.
Therefore spider venoms are particularly rich in insect-selective neurotoxins that target ion channels (see below and the review by King in this edition) and to a lesser extent affect neurotransmitter exocytosis (e.g. latroinsectotoxins; see the accompanying review by Rohou et al. in this edition). Other targets include ionotropic glutamate and acetylcholine receptors, although in these cases the polyamine-containing toxins that modulate their activity are not selective for insect targets and exert potent actions on vertebrate receptors (see reviews by (Mellor and Usherwood, 2004) and (Adams, 2004)).

Many of these peptides are selectively insecticidal and are now being investigated for their possible use as bioinsecticidal agents for the control of phytophagous or hematophagous pests, or insect vectors of new or re-emerging disease (Tedford et al., 2004b). The focus of this review is the discovery, processing, structure and function of these insect-selective spider venom neurotoxins, specifically those targeting the Na\textsubscript{v} channel. In particular, it will detail the site and mechanism of their action, the molecular determinants for their pharmacology, and discuss the application of these peptides in the development of novel insecticides.

2. Sodium channel structure and function

Na\textsubscript{v} channels are transmembrane proteins that provide the current pathway for rapid depolarization of excitable cells to initiate action potential generation (Hodgkin and Huxley, 1952). Their structure comprises principally of a single pore-forming ~2000-residue glycoprotein \(\alpha\)-subunit in eukaryotic Na\textsubscript{v} channels (Catterall, 2001). The \(\alpha\)-subunit is composed of four homologous, but non-identical, domains (I–IV) connected by cytoplasmic linkers (Fig. 1A) (Catterall, 2000; Morgan et al., 2000; Yu and Catterall, 2003). Each of these domains contains six putative transmembrane segments (S1–S6). The four domains fold together in a clockwise orientation, where domains I and IV are brought into close proximity, to form the outer pore vestibule and the selectivity filter. This is created by the S5-S6 linker loops from each domain that form re-entrant pore loops (P) that dip into the transmembrane region of the protein (Catterall, 2000; Li et al., 2001) (Fig. 1A). The S4 segments, which are the most conserved segments, have positively charged amino acids (Arg or Lys) at intervals of three residues and transport gating charges outward thus acting as voltage sensors to initiate voltage-dependent activation by moving outward under the influence of changes in the electric field (Stuhmer et al., 1989; Chen et al., 1996; Yang et al., 1996; Chanda and Bezanilla, 2002; Cestèle et al., 2006). Sodium channel inactivation is mediated by a short intracellular loop connecting domains III and IV, containing the key hydrophobic amino acid residues IFM (West et al., 1992) (Fig. 1A). The \(\alpha\)-subunit
is also associated with one or two smaller auxiliary subunits (β1, β2, β3 and/or β4) of approximately 30 kDa that are required for normal kinetics and voltage-dependence of gating but are not required for ion flux, ionic selectivity and pharmacological modulation (Schreibmayer et al., 1994; Isom et al., 1995; Yu et al., 2003) (Fig. 1A).

Figure 1 Here

3. Neurotoxin receptor sites: targets for molecular probes, drugs and insecticides

The Na$_v$ channel is the primary molecular target of numerous therapeutic drugs (e.g. local anesthetics, anticonvulsants and antiarrhythmics) and insecticides (eg. pyrethroids, DDT, dihydropyrazoles, oxadiazines and N-alkylamides). However, much of its structure and function has been elucidated using guanidinium, peptide and small alkaloid toxins of various plant and animal origins. These molecular probes bind with at least seven identified neurotoxin binding sites, referred to as neurotoxin receptor sites 1-7 (Table 1 and Fig. 1B; for recent reviews see (Gordon, 1997b; a; Cestèle and Catterall, 2000; Tan et al., 2005) and alter voltage-dependent activation, conductance and inactivation. Ligands are associated with a receptor site if they compete in radioligand competition binding assays, often with specific allosteric interactions with other sites, or elicit similar electrophysiological effects. Four neurotoxin sites that bind peptide toxins exist on Na$_v$ channels.

Site 1, located on the extracellular surface of the pore, binds the peptide toxins Tx1 from the spider *Phoneutria nigriventer* and the μ-conotoxins from *Conus* spp., as well as the guanidinium alkaloids tetrodotoxin (TTX) and saxitoxin (STX) (Fig. 1B). These toxins physically occlude the conduction pathway (Narahashi et al., 1964; Ritchie and Rogart, 1977; Noda et al., 1989; Terlau et al., 1991; Chahine et al., 1995; Dudley et al., 1995; Chahine et al., 1998; Martin-Moutot et al., 2006). Site-3 toxins, including the classical scorpion α-toxins, Type 1 and Type 2 sea anemone toxins, and select spider toxins bind to the S3-S4 extracellular loop in domain IV (Rogers et al., 1996) as well as unidentified residues in S5-S6 linkers in domains I and IV (Tejedor and Catterall, 1988; Thomsen and Catterall, 1989) (Fig. 1B). They slow or remove channel inactivation by preventing the normal outward movement of the IVS4 transmembrane segment during channel gating, trapping it in the inward position (Rogers et al., 1996; Nicholson et al., 1998; Sheets et al., 1999). This site has also been shown to have complex allosteric interactions with site-2, which binds several lipid soluble alkaloid toxins such as batrachotoxin and veratridine, and site-5 which binds the cyclic polyether toxins brevetoxin and
ciguatoxin (Cestèle and Catterall, 2000; Zlotkin et al., 2000) (Table 1). Site-4 toxins include the scorpion $\beta$-toxins that bind to the S1-S2 and S3-S4 linkers in domain II and facilitate channel activation by trapping the S4 segment in its outward position and shifting the voltage-dependence of activation to more hyperpolarized potentials (Jaimovich et al., 1982; Meves et al., 1982; Wang and Strichartz, 1983; Vijverberg et al., 1984; Jonas et al., 1986; Marcotte et al., 1997; Cestèle et al., 1998; Cestèle et al., 2006) (Fig. 1B). Site-3 and site-4 toxins mainly increase the open probability of Na$_V$ channels and inhibit gating transitions into closed states and are thus classified as 'gating-modifiers'. The modification in gating results from either an inhibition of deactivation (eg. scorpion $\beta$-toxins) or inhibition of the transition to the fast-inactivated state of the channel (eg. scorpion $\alpha$-toxins). Nevertheless, the basis for this classification is under review following the characterization of the actions of $\delta$-palutoxins (see section 9.3). Finally, site-6 binds the $\delta$-conotoxin TxVIA which slows channel inactivation but shows different allosteric modulation to site-3 (Fainzilber et al., 1994) (Table 1).

4. Insect vs. mammalian voltage-gated Na$_V$ channels: variation on a theme?

The domain structure and function as well as the presence of allosterically coupled neurotoxin receptor sites means that the insect Na$_V$ channel closely resembles its mammalian counterpart. Na$_V$ channels have been conserved across evolution and therefore it is not surprising to find that similar neurotoxin receptor sites are found on insect and mammalian neuronal Na$_V$ channels, in addition to other voltage-gated channels such as Ca$_V$ channels (Wicher et al., 2001). However insect and mammalian Na$_V$ channels are distinguishable pharmacologically due to the selective actions of four groups of agrochemical insecticides such as DDT and its analogues, pyrethroids, N-alkylamides, oxadiazines and dihydropyrazoles (Sattelle and Yamamoto, 1988; Soderlund and Bloomquist, 1989; Salgado, 1992; Bloomquist, 1996; Narahashi et al., 1998; Zlotkin, 1999; Narahashi, 2000; Raymond-Delpech et al., 2005) as well as a growing range of insect-selective Na$_V$ channel neurotoxins derived from arachnid venoms (see section 9). To date, nine mammalian Na$_V$ channels (Na$_V$1.1-1.9) have been cloned, functionally expressed and characterized (Goldin et al., 2000). It appears that the structural, functional and pharmacological diversity of mammalian Na$_V$ channels is achieved primarily through expression of distinct Na$_V$ channel genes. Indeed, a variety of non-insecticidal spider toxins are proving useful in the study of molecular differences in Na$_V$ channel subtypes including ceratotoxins 1, 2, and 3 from Ceratogyrus cornuatus, and phrixotoxin 3 for Phrixotrichus auratus. These toxins block conductance and shift the voltage-dependence of activation toward more positive values, without affecting
inactivation (Bomsans et al., 2006). Interestingly certain toxins show that subtle differences in their molecular surface can cause changes in Na_v channel subtype specificity. With ceratotoxins, a single amino acid mutation from Tyr^{32} in ceratotoxin 2 to Asp^{32} in ceratotoxin 1 completely blocks actions on the Na_{1.3} channel subtype. Presently, the exact site of action of these toxins on the Na_v channel remains speculative, but probably is associated with site-4.

At least 18 genes encoding Na_v channels have been cloned from invertebrate species, most of which have not been functionally expressed (Ramaswami and Tanouye, 1989; Goldin, 2002). Initial studies identified two Na_v channel α-subunit-like genes from insects, DSC1 and para. DSC1 was originally isolated from a Drosophila genomic DNA library using an eel Na_v channel cDNA probe (Salkoff et al., 1987), and para was identified using temperature-sensitive paralysis phenotypes displayed by mutant alleles in Drosophila (Loughney et al., 1989; Ramaswami and Tanouye, 1989; Thackeray and Ganetzky, 1994). More recently, orthologous genes such as Musca domestica housefly Vssc1 (housefly para channel), as well as BSC1 and BgNa_v1-1 (formerly para^{CSMA}) from the German cockroach (Blattella germanica) have also been identified (Dong, 1997; Liu et al., 2001) and functionally expressed in Xenopus laevis oocytes (Feng et al., 1995; Smith et al., 1997; Warmke et al., 1997; Tan et al., 2002a; Tan et al., 2002b; Soderlund and Knipple, 2003; Tan et al., 2005). However, recently BSC1 has been found to encode for a Ca^{2+}-selective channel with different functional properties compared to Na_v channels (Zhou et al., 2004). An auxiliary β-subunit for the insect Na_v channels, the product of the Drosophila temperature-induced paralysis (TipE) locus, has also been identified (Feng et al., 1995) and appears to be important for trafficking the para α-subunit from the endoplasmic reticulum to the plasma membrane (Moore et al., 2001).

The deduced polypeptide primary sequence of para is 67% identical to the rat brain Na_{1.2} channel α-subunit (Loughney et al., 1989) and has four internally homologous domains similar to those conserved in all other Na_v channels (Guy and Conti, 1990; Catterall, 1992). Unlike its mammalian counterparts, the primary transcript from the para gene undergoes a complex pattern of alternative splicing and RNA editing at least five sites that potentially generates over 100 different tissue/cell type-specific and functional distinct variants of insect Na_v channel isoforms (Thackeray and Ganetzky, 1994; O’Dowd, 1995; Tan et al., 2002b; Liu et al., 2004; Song et al., 2004).

The structural differences between mammalian and arthropod Na_v channels is also reflected in differences in the allosteric modulation of neurotoxin receptor sites and insect Na_v channels are often more sensitive to the actions of these neurotoxins (Warmke et al., 1997). Firstly, veratridine (site-2) induces a positive allosteric modulation of receptor site-3 on rat brain and locust Na_v channels (Ray et
al., 1978; Jover et al., 1980; Gordon and Zlotkin, 1993), but has no effect on the binding of (-scorpion toxins to cockroach Na_v channels (Cestèle et al., 1995). In addition, brevetoxin (site-5) induces a negative allosteric modulation of site-3 on rat brain sodium channels, a positive modulation of locust Na_v channels and has no action on cockroach Na_v channels (Cestèle et al., 1995). Therefore, site-3 toxins bind to homologous but non-identical receptor sites on rat brain and insect Na_v channels and can even distinguish between Na_v channels in different insect orders (i.e. Dictyoptera vs. Orthoptera; (Gilles et al., 2002a)).

5. Are sodium channels good targets for the development of biopesticides?

The insect Na_v channel presently represents a long-term potential target for the development of novel insect-selective biopesticides. This arises because (i) it has been shown, thus far, to possess a large number of binding sites, currently far more than other insecticidal toxins targeting Ca_v and K_v channels, thus potentially providing for a great diversity of potential insecticidal targets, (ii) it is distinct from mammalian Na_v channels, as revealed by the use of spider and other sea anemone and arachnid toxins, and (iii) its pharmacological diversity as exhibited by the allosteric coupling of these binding sites provides additional flexibility (Zlotkin, 1999). Insect-selective spider toxins targeting the Na_v channel have now been described and bind with three of the major peptide toxin target sites (see section 8 below). These validate sites 1, 3 and 4 as potential insecticide targets.

Nevertheless, many insects have already developed resistance to insecticides targeting Na_v channels, as exemplified by the knockdown resistance (termed kdr and super-kdr) mutations that confer resistance to pyrethroids and other chemical insecticides (reviewed in (Vais et al., 2001; Soderlund and Knipple, 2003). Despite this, the Na_v channel can continue to be exploited as an insecticide target as, kdr mutants are actually more sensitive (termed negative cross-resistance) to certain chemical insecticides and peptide toxins that act at remote sites on the channel (Elliott et al., 1986; McCutchen et al., 1997; Zlotkin et al., 1999). However, there is no reason that the Na_v channel will prove to be a better target for future insecticide development than other voltage-gated ion channels such as K_v or Ca_v channels (see review by King in this edition). Given their structural homology with Na_v channels, insect Ca_v channels are likely to provide a similarly diverse array of potential binding sites for insecticide development, particularly since there are at least three distinct classes of Ca_v channels identified in the insect nervous system (Jeziorski et al., 2000).
6. Spider venoms: combinatorial peptide libraries rich in insecticidal compounds

Spiders have developed a combinatorial peptide library of insecticidal peptides in their venom, which they rely upon to kill or paralyze their prey. This library of toxins has been optimized over a period of 300-400 million years, resulting in an insecticidal mixture of the most successful variants. Over this time scale, spiders have evolved various cysteine frameworks as structural scaffolds to synthesise these libraries. Hypermutation of the mature toxin sequence has assisted in the evolution and optimisation of toxins with functional residues (Sollod et al., 2005). Maintenance in the venom of a mini-library of toxin variants most probably enables the spider to target slightly altered versions of the same receptor in different insects (Gilles et al., 2002b), and perhaps combat the development of natural resistance in the prey. As a result, spider venom contains pre-optimized insecticidal toxins, which are readily available for investigation to isolate any appropriate candidates (toxins) for insertion into baculovirus vectors, or design of chemical insecticides. Spider venom peptides, like those from marine cone snails and sea anemones, are translated mostly as prepropeptides that undergo post-translational modification to yield the mature toxin (Sollod et al., 2005). It appears that during evolution families of toxins within spider venoms underwent hypermutation in the mature peptide region while conserving the basic disulfide framework. This cystine framework appears to be associated with a specific signal sequence. However the signal peptide was conserved since its role was, most likely, to direct the precursor to a specific secretory pathway, thereby ensuring correct peptide folding. The specific role(s) of the propeptide region, however, is still not understood; it may play a role directing proteolytic processing of the propeptide and in signalling post-translational modifications such as N-terminal pyroglutamate formation, palmitoylation, C-terminal trimming and amidation previously described in spider toxins (Bodi et al., 1995; Corzo et al., 2003; Pimenta et al., 2005; Wen et al., 2005). Curiously, however, in the case of the larger MIT-like ACTXs (Wen et al., 2005) and latrotoxins (Ushkaryov et al., 2004), this N-terminal propeptide is absent.

7. Methodological approaches in the isolation of insecticidal spider venom peptides

Several different methodological approaches have led to the isolation of novel insecticidal spider peptide toxins (for a more extensive overview see (Escoubas and Rash, 2004)). The most commonly used approach has been the use of insect bioassays to drive venom fractionation using acute toxicity testing via injection assays or isolated organ bath screening. The weakness with this approach is that numerous toxins remain uncharacterized in terms of target and mode of action eg. lasiotoxins 1 and 2 [LpTx1 and 2], *Eurypelma* toxins [ESTx1 and 2], and covalitoxin II (Escoubas and Rash, 2004).
and atracotoxin-Hvf17 (Szeto et al., 2000b; Wen et al., 2005). Also the potential array of targets for a novel toxin is too vast to be comprehensively screened. This is compounded by the variable susceptibility of insect targets across the 30 insect orders particularly differences between Dictyoptera and Orthoptera (Gordon et al., 1996a; Gilles et al., 2000; Gilles et al., 2002b; Bosmans et al., 2005). A more recent approach has been to study all components of a single venom, or venoms of closely-related species, and characterize their mode of action in target-oriented bioassays. This has been the approach taken with the atracotoxins (ACTXs) from Australian funnel-web spiders and huwentoxins (HWTXs) from Chinese bird spiders where several families of toxins that modulate voltage-gated Na\(^+\) (Na\(_v\)), Ca\(^{2+}\) (Ca\(_v\)), and Ca\(^{2+}\)-activated K\(^+\) channels have been described. The limitation of this approach is that it does not always yield the most active toxin for a specific target and, again, many toxins remain uncharacterized. Currently, a number of groups are using a systematic approach employing screening assays using cloned ion channels expressed in *Xenopus* oocytes or mammalian cell lines. Such methods have provided high-affinity and often highly selective ligands that block or modulate ion channel subtypes for which there were no previous pharmacological tools (eg. PcTx1 as a blocker of ASIC channels; (Escoubas et al., 2003)). Unfortunately, this approach is limited if the insecticidal target has not been previously validated or if the target has not yet been cloned, which for invertebrate ion channels is often the case. This limits discovery of ligands to only those with affinity for known target subtypes. Accordingly, methodologies centered around a combination of the last two approaches are likely to be the most successful.

8. Structural organization of spider venom peptides: variations on an ancestral fold

The limited number of known structures of insecticidal spider toxins targeting the Na\(_v\) channel precludes a detailed analysis of their structure-function relationships. Nevertheless all appear to be compact globular proteins possessing several disulfide bridges that increase their *in vivo* stability. These peptides, like the vast majority of all spider venom peptides, contain a disulfide pseudo-knot which places them in a class of toxins and inhibitory polypeptides with an ‘inhibitor cystine-knot’ (ICK) motif (Norton and Pallaghy, 1998). This structural motif is normally exemplified by a triple-stranded, antiparallel β-sheet stabilized by disulfide bridges (Fig. 2Ba). Since not all ICK peptides exhibit the N-terminal β-sheet (β1 in Fig. 2Ba), a modified definition comprising ‘an antiparallel β-hairpin stabilized by a cystine-knot’ without a mandatory third β-sheet has been proposed (Wang et al., 2000; Tedford et al., 2004b) (Fig. 2A). The consensus sequence for the ICK motif is currently -C\(_i\)X\(_{3-7}\)-C\(_{II}\)X\(_{3-8}\)-C\(_{III}\)X\(_{6-7}\)-C\(_{IV}\)X\(_{1-6}\)-C\(_v\)X\(_{3-13}\)-C\(_{V1}\)- where X is any amino acid. The three disulfide bridges and
intervening backbone form a pseudo-knot consisting of a ring (C_I-C_IV, C_{II}-C_V) penetrated by a third disulfide bridge (C_{III}-C_{VI}) (see Fig. 2C). This is similar to marine molluscs and, importantly, includes all spider toxins targeting vertebrate or insect Na_v channels whose disulfide-bonding pattern has been determined to date. However, within this fold-class, the biological activities of other ICK toxins are quite diverse with activity at Ca_v, K_v, proton-gated and mechanosensitive channels (see (Nicholson, 2006) and haemagglutination activity on red blood cells (eg. ShL-I; (Liang, 2004)). This highlights that different biological functions are often grafted onto the same, or similar, structural scaffolds (King et al., 2002). Nevertheless, the cystine-knot no doubt contributes to the high stability and resistance to proteases of these spider toxins, possibly reducing degradation in the venom gland and also in the prey following envenomation and provides an effective scaffold for the design of novel biopesticides.

Figure 2 Here

9. Arthropod Na_v channels as molecular targets of insect-selective spider toxins

9.1. Pore blocking toxins: potential site-1 ligands

A family of 33-35 residue toxins with three disulfides known as the hainantoxins and huwentoxins have been isolated from the venom of the Chinese bird spider Ornithoctonus spp. (formerly Selenocosmia) (Mygalomorphae: Theraphosidae) and have been found to target the Na_v channel (Fig. 3). Importantly, hainantoxin-I (HNTX-I) from Ornithoctonus hainana, displays a 15-fold selectivity for the para/tipE insect Na_v channel over the rat Na_v1.2/β1 channel, with no effect on a variety of other Na_v channels (Li et al., 2003). It is similar in structure and function to HWTX-IV from Ornithoctonus huwena and HNTX III-V. All members of this family, except HNTX-I, block TTX-sensitive Na_v channel currents (I_{Na}) of adult dorsal root ganglion (DRG) neurons with no significant effect on TTX-resistant Na_v or Ca_v channel currents. This action occurs in the absence of any alterations in channel inactivation kinetics or the voltage dependence of channel activation, but is associated with a shift in the voltage dependence of steady-state inactivation (Peng et al., 2002; Li et al., 2003; Xiao and Liang, 2003a; Xiao and Liang, 2003b). It has been claimed that this group of polypeptides are the first family of spider toxins to selectively block Na^+ conductance via an interaction with site-1 of the Na_v channel. However, competition radioligand binding studies using [³H]-STX to confirm this interaction are still awaited. Thus the site could be distinct from site-1, but near the pore, or potentially at a remote site that allosterically leads to a conformational change in the channel protein.
resulting in a block of ion conductance. Nevertheless, HNTX-I represents the first insect-selective spider toxin interacting with site-1 or a novel site on the Na\textsubscript{v} channel. Therefore these peptides represent the first family of spider toxins to selectively block Na\textsuperscript{+} conductance and, in the case of HNTX-I, the first insect-selective toxin to block the Na\textsubscript{v} channel.

The NMR structures of several of these toxins have also been determined and comprise of a double-stranded antiparallel \( \beta \)-sheet motif (Fig. 4 left-hand panels). By synthesizing various alanine mutants, it has been determined that the key residues responsible for the affinity of HNTX-IV for the Na\textsubscript{v} channel are most likely Lys\textsuperscript{27}, Arg\textsuperscript{29}, His\textsuperscript{28}, Lys\textsuperscript{32}, Phe\textsuperscript{5}, and Trp\textsuperscript{30} (Li et al., 2004), residues that appear to be conserved in HWTX-IV (Fig. 3). Interestingly His\textsuperscript{28} is substituted by the negatively charged Asp\textsuperscript{26} in HNTX-I (Fig. 4 right-hand panels) providing a possible molecular basis for the selectivity of HNTX-I for the insect Na\textsubscript{v} channel.

9.2. Spider toxins interacting with neurotoxin site-3: gating modifiers of inactivation

Spider envenomation in Brazil is mostly caused by bites from the South American ‘armed’ spider *Phoneutria nigriventer* (Araneomorphae: Ctenidae). Several neurotoxic crude fractions (PhTx1, 2, 3 and 4) have been isolated from the venom (Cordeiro et al., 1995) and a novel toxin, Tx4(6-1), a 48-residue polypeptide with 5 disulfide bonds, has been isolated from fraction PhTx4 (Figueiredo et al., 1995) (Fig. 3). It is not toxic to mammals but is lethal to a variety of insects. Electrophysiological experiments using isolated cockroach axons found that the toxin prolongs action potential duration also via a slowing of Na\textsubscript{v} channel inactivation. This occurred in the absence of alterations to the voltage dependence of Na\textsubscript{v} channel activation or steady-state inactivation. Patch clamp experiments on Na\textsubscript{1.2} and Na\textsubscript{1.4} channels expressed in *Xenopus oocytes* revealed that Tx4(6-1) failed to alter any aspects of Na\textsubscript{v} channel gating or kinetics indicating that this toxin appears to be insect-selective (de Lima et al., 2002). Given these insect-selective actions on site-3 it is not surprising that the toxin has been found to compete with the scorpion \( \alpha \)-like toxin Bom IV for site-3 on insect Na\textsubscript{v} channels (de Lima et al., 2002).

Several peptide toxins have been isolated from another mygalomorph spider, the Japanese funnel-web *Macrothele gigas* (Mygalomorphae: Hexathelidae), which display toxicity to mammals or insects. Of these toxins, five have been shown to displace radiolabelled neurotoxin binding to the Na\textsubscript{v}
channel. Magi 2, a neurotoxin from the Japanese funnel-web spider *Macrothele gigas* has been found to induce paralysis in insects but lacks mammalian toxicity (Corzo et al., 2003). It displays low sequence homology with known toxins but it does share sequence homology with Magi 1, a peptide that fails to exhibit any overt toxicity in insects or mammals (Fig. 3). Magi 2 has been shown to displace the insect site-3 ligand $^{125}\text{I}-\text{Lqh\alpha IT}$ from cockroach synaptosomes with a $K_i$ of 21 nM, whereas the $K_i$ for displacement by Magi 1 is around 83-fold higher. Importantly, they fail to inhibit binding of radiolabelled neurotoxins to site-4 on insect, or site-3, 4 or 6 on mammalian Na$_v$ channels. Like other site-3 spider toxins, Magi 2 awaits delineation of its structure-activity relationships in order to reveal the molecular determinants of its specificity for the insect sodium channel. Thus Tx4(6-1) together with Magi 2 represents a growing family of insect-selective spider neurotoxins targeting site-3 on the insect Na$_v$ channel.

9.3. Spider toxins interacting with neurotoxin site-4: gating modifiers of activation

Other insect-selective toxins interact with Na$_v$ channel site-4 by acting as gating modifiers of activation. $\delta$-Palutoxins ($\delta$-PaluITs) from the spider *Paracoelotes luctuosus* (Araneomorphae: Amaurobiidae) are a family of four 36-37 residue peptides that selectively modulate insect Na$_v$ channels (Corzo et al., 2000) (Fig. 3). Using the isolated cockroach axon preparation and cloned para/tipE insect Na$_v$ channels expressed in *Xenopus oocytes*, they have been shown to slow insect Na$_v$ channel inactivation with no shift in the voltage dependence of activation. They also have no effect on Na$_v$1.2/$\beta$1 channels at concentrations up to 10 $\mu$M (Ferrat et al., 2005). This action is similar to site-3 neurotoxins such as the scorpion $\alpha$-insect toxin, Lqh\alpha IT. Despite this they have been shown to displace the site-4 excitatory scorpion $\alpha$-toxin, Bj-xtrIT, from binding on cockroach membranes and fail to displace Lqh\alpha IT binding (Corzo et al., 2005). In reciprocal experiments, Bj-xtrIT and the depressant scorpion $\alpha$-toxin LqhIT2 also displaced $^{125}\text{I}$-$\delta$-PaluIT2 binding (Corzo et al., 2005). Thus $\delta$-PaluITs represent the first spider toxins that definitively bind to site-4 on insect Na$_v$ channels but modulate Na$_v$ channel inactivation. To date, only scorpion $\alpha$-toxins have been shown to compete with this site. The 3D structures of $\delta$-PaluIT1 and -IT2 have been recently determined by NMR spectroscopy and, like all other spider toxins targeting the Na$_v$ channel, found to belong to the ICK structural family (see Fig. 5Aa). $\delta$-PaluIT1 and $\delta$-PaluIT2 contain double and triple-stranded anti-parallel $\beta$-sheets, respectively. Alanine scanning mutagenesis experiments reveal that the putative insectophore of $\delta$-PaluIT2 (Corzo et al., 2005) shares similarity with the bipartite bioactive surface of Bj-xtrIT (Cohen et al., 2004) despite
different protein scaffolds (see Fig. 5Aa). The differences in the mode of action of δ-PaluIT toxins and scorpion β-toxins provides a novel perspective about the structural relatedness of receptor sites 3 and 4 which, to-date, have been considered to be topologically distinct, and suggest that receptor site-4 is an extended macrosite. Thus these toxins reveal that modulation of inactivation can be achieved by binding to a site, until now, thought to be associated with effects on channel activation.

Araneomorph spider venoms have also been found to contain insect-selective neurotoxins. The μ-agatoxins from the venom of the American funnel-web spider *Agelenopsis aperta* (Araneomorphae: Agelenidae) are a family of six 36–37 residue peptides containing four disulfide bridges (Skinner et al., 1989). They show high sequence homology to the curtatoxins from the related agelenid spider *Hololena curta* (Stapleton et al., 1990) (Fig. 3). Little data is available as to their actions but it is known that the μ-agatoxins are insect-selective neurotoxins that cause a convulsive paralysis in insects. This action is correlated with repetitive firing in the terminal branches of the insect motor axons resulting in a marked increase in spontaneous neurotransmitter release of the fly *Musca domestica* (Adams et al., 1989). This correlates with a ~30 mV hyperpolarizing shift in the voltage-dependence of Na, channel activation causing channels to open at, or close to, the resting membrane potential (Cohen et al., 1993; Norris et al., 1995). The increase in open channel probability leads to repetitive firing and consequently increased Ca entry into nerve terminals resulting in the increased frequency of miniature excitatory junctional potentials. This action is analogous to that reported for scorpion β-toxins (Wang and Strichartz, 1983) and therefore it is likely that this family targets site-4, although this awaits further radioligand binding studies. However μ-agatoxins also slow Na, channel inactivation in insect motoneurons from *Heliothis virescens* (Cohen et al., 1993; Norris et al., 1995) an action shared by δ-PaluIT toxins with whom they share considerable sequence homology (Fig. 3). The similarities in primary structure and pharmacology of these toxins provide further support for the hypothesis that the insect site-4 is a macrosite, which may be allosterically linked to channel inactivation. Future experiments using these above spider toxins may reveal the mechanism of this connection.

Figure 5 Here

9.4. Toxins interacting with unidentified sites on the Na, channel

While a number of spider toxins have been determined by voltage-clamp electrophysiology to interact with a known neurotoxin receptor site on the Na, channel, several insect-selective toxins are
still awaiting identification of their target site. A family of 56-61 residue insecticidal polypeptides, DTX9.2, 10 and 11 (Fig. 3) have been isolated from the venom of the primitive weaving spider *Diguentia canities* (Araneomorphae: Diguentidae) (Krapcho et al., 1995; Bloomquist et al., 1996). These insect-selective neurotoxins cause progressive spastic paralysis in tobacco budworm larvae without effects on mice following intraperitoneal or intracerebroventricular injection. DTX9.2 causes repetitive excitatory postsynaptic potential discharges in housefly larvae neuromuscular and sensory nerve preparations and a depolarization of cockroach axons, actions that were are blocked by TTX indicating that the Na\textsubscript{v} channel is the cellular target (Bloomquist et al., 1996). However preliminary radioligand binding studies revealed only a partial inhibition of $^{125}$I-AahIT binding to site-3 on housefly head membranes (Bloomquist et al., 1996). Further voltage-clamp and binding studies are required to determine the precise target on insect Na\textsubscript{v} channels, but it is unlikely that DTX9.2 interacts with site-3.

Funnel-web spider venoms have been rich sources of toxins targeting Na\textsubscript{v} channels, in particular the δ-atracotoxins (Nicholson et al., 2004). Venom of the funnel-web spider *Hadronyche infensa* Orchid Beach also contains a 38-residue polypeptide, ACTX-Hi:OB4219, containing four disulfide bonds but with no significant homology to the δ-atracotoxins (Fig. 3). NMR spectroscopy reveals a triple-stranded antiparallel β-sheet with ICK motif and, despite the isolation of a single native homologous product by HPLC, the polypeptide possesses two conformers arising from cis-trans isomerization of Pro\textsuperscript{30} (Rosengren et al., 2002). This isomerization has also been reported for the NMR spectra of μ-Aga IV, although a full determination of the structure was not possible (Omecinsky et al., 1996). Indeed ACTX-Hi:OB4219 shares an identical cysteine framework and loop sizes to the μ-agatoxins, as well as other toxins that target site-4 such as δ-PaluITs and curtatoxins. Despite a similar fold to μ-Aga I and to a lesser extent δ-PaluIT2 (Fig. 5Aa-b), ACTX-Hi:OB4219 shares limited sequence identity with these toxins. Therefore, while ACTX-Hi:OB4219 may target Na\textsubscript{v} channels, these differences in the primary structure require electrophysiological or binding studies to definitively determine the target of ACTX-Hi:OB4219.

In addition, an insect-selective neurotoxin oxytoxin1 from *Oxyopes kitabensis*, is a 69-residue peptide with a molecular mass of 8059.2 Da and five predicted disulfide bridges (Corzo et al., 2002). OxyTx1 has limited sequence identity (33%) to Tx4(6–1) from *P. nigriventer*, an insecticidal toxin that is non-toxic to mice (Figueiredo et al., 1995) and slows down the inactivation of the Na\textsubscript{v} channel in insect CNS via binding to receptor site-3 (de Lima et al., 2002). Similarly to Tx4(6–1), OxyTx1 is an insecticidal toxin that is non-toxic to mice up to 1 g/20 g mouse, but further investigations are required to determine it the target of OxyTx1 are insect sodium channels.
10. Toxins with non-selective actions on voltage-gated ion channels: promiscuous toxins

The promiscuous activity of certain spider toxins on multiple voltage-gated ion channels may arise due to common structural elements shared between these channels, particularly Na\(_v\) and Ca\(_v\) channels. These toxins may recognize a common domain or motif present in voltage-gated ion channels that has a highly conserved three-dimensional structure. For example it has been proposed that hanatoxin and \(\omega\)-grammotoxin SIA, two related protein toxins found in the venom of the Chilean rose tarantula (Grammostola spatulata, now G. rosea), both recognize a voltage-sensing domain of K\(_v\) and Ca\(_v\) channels (Li-Smerin and Swartz, 1998).

It has been previously noted that peptide toxins can exert their actions both within and across voltage-gated ion channel families. For example Magi 3 from Macrothele gigas is an insect-selective neurotoxin that causes a reversible paralysis in insects but fails to display any signs of toxicity in mammals. It has also been shown to partially inhibit \(^{125}\)I-Lqh\(_{\alpha}\)IT binding to cockroach synaptosomes (Corzo et al., 2003). The primary structure of Magi 3, however, shows significant homology to PlTx-II from the North American spider Plectreurys tristis (Araneomorphae: Plectreuridae) a known blocker of Ca\(_v\) channels (Branton et al., 1987). Thus while Magi 3 displays some limited affinity for the insect Na\(_v\) channel its major target is more likely to be an insect Ca\(_v\) channel. This dual activity on Na\(_v\) and Ca\(_v\) channels has been reported for the P/Q-type blocker \(\omega\)-agatoxin IVA which also decreased TTX-sensitive \(I_{\text{Na}}\) amplitude, enhanced \(I_{\text{Na}}\) decay and led to a slower recovery from Na\(_v\) channel inactivation in cockroach DUM neurons (Wicher and Penzlin, 1998). This effect has been noted recently to occur with the insect-selective calcium channel blocker \(\omega\)-ACTX-Hv1a (Y. Chong and G.M. Nicholson, unpublished observations).

11. Molecular determinants of phyla-specificity: use of non-selective toxins

Spider \(\delta\)-toxins from funnel-web (Hadronyche and Atrax spp.) and eastern mouse spiders (Missulena bradleyi) are lethal toxins, responsible for the major symptoms of human envenomation. This family of 42-residue peptides, the \(\delta\)-atracotoxins and \(\delta\)-MSTX-Mb1a, have been shown to target the Na\(_v\) channel (for a overview see (Nicholson et al., 2004)). Importantly \(\delta\)-ACTXs are, in addition to being mammalian toxic, also insecticidal. Both \(\delta\)-ACTX-Ar1a and \(\delta\)-ACTX-Hv1a are lethal by lateroventral injection into crickets, showing similar signs of delayed contractile paralysis as the anti-insect scorpion \(\alpha\)-toxin Lqh\(_{\alpha}\)IT (Eitan et al., 1990; Little et al., 1998a). These neurotoxic actions on
insects led researchers to investigate the effects of δ-atracotoxins on insect Na\(_v\) channels. In isolated giant axon and dorsal unpaired median neurones of the cockroach *Periplaneta americana*, δ-ACTX-Hv1a modified the action potential by prolonging the repolarization phase, causing the development of spontaneous plateau action potentials. Under voltage-clamp conditions, these alterations in neuronal excitability were found to be due to a slowing of Na\(_v\) channel inactivation and a shift in the voltage-dependence of activation towards more negative potentials (Grolleau et al., 2001). These actions are very similar to LqhαIT (Eitan et al., 1990) and almost identical to that observed in mammalian preparations (Nicholson et al., 1994; Nicholson et al., 1998; Szeto et al., 2000a; Nicholson et al., 2004).

In contrast to the phyla-specific actions of anti-mammalian scorpion α-toxins, radioligand binding experiments revealed that δ-atracotoxins inhibit \(^{125}\)I-labelled LqhαIT binding to cockroach neuronal membranes (Little et al., 1998a; Gilles et al., 2002a). Therefore δ-atracotoxins are unique in that they bind with equal high affinity to site-3 of both rat brain and cockroach Na\(_v\) channels (Little et al., 1998a; Little et al., 1998b; Gilles et al., 2002a). Notably, however, δ-ACTX-Hv1a exhibits a low binding affinity to locust sodium channels (Gilles et al., 2002a). Thus unlike scorpion toxins, which are only capable of differentiating between mammals and insects, δ-atracotoxins differentiate between insect Na\(_v\) channels from different insect orders (i.e. Dictyoptera vs. Orthoptera). Structural differences between the two types of insect Na\(_v\) channels have been previously inferred from allosteric modulations of LqhαIT binding. For example, brevetoxin (site-5) and veratridine (site-2) have been shown to increase the binding of LqhαIT to locust but not cockroach Na\(_v\) channels (Gordon and Zlotkin, 1993; Cestèle et al., 1995; Gordon et al., 1996b). In addition, it has been shown that the α-like scorpion toxin Lqh-III, which binds to locust and cockroach site-3 with equal affinity, preferentially lost its binding capacity to locust sodium channels upon iodination (Gilles et al., 2000). The ability of site-3 to differentiate between such subtle structural alterations on Lqh-III (addition of an iodine atom) and the differential binding of δ-atracotoxins suggests structural differences at the binding site between Na\(_v\) channels from the two insect orders. Hence, employing various toxin probes can expose subtle differences at receptor site-3. Unfortunately, the structure-function relationships of these spider toxins have not been determined directly. The presence of four disulfide bonds has severely limited the efficient production of synthetic or recombinant δ-atracotoxins in order to assess the effect of mutations to ascertain the toxin pharmacophore. Importantly, they do not share sequence homology or even fold homology with anti-mammal and anti-insect scorpion α-toxins such as Aah-II or LqhαIT or even sea anemone toxins. Nevertheless, attempts have been made to indirectly identify critical residues involved
in δ-atracotoxin binding to site-3 based on knowledge of the binding site on the Na\(_v\) channel or from mutagenesis studies of related site-3 toxins. These studies indicate that a number of toxin residues provide either a complementary surface to the residues identified in the S3-S4 loop of domain IV (Fletcher et al., 1997a), or are topologically related to key residues forming the likely pharmacophores of scorpion toxins and sea anemone toxins (Gilles et al., 2002a). These comprise of the residues highlighted in Figure 5B.

Recently a homologous δ-atracotoxin was isolated from the venom of the Blue Mountains funnel-web spider \textit{H. versuta} (Szeto et al., 2000a). This 42–residue peptide, designated δ-ACTX-Hv1b, shows 67\% sequence identity with δ-ACTX-Hv1a previously isolated from the same spider (Szeto et al., 2000a). δ-ACTX-Hv1b is unique amongst the δ-atracotoxins in that it lacks insecticidal activity and shows a 15- to 30-fold reduction in mammalian activity compared to δ-ACTX-Hv1a/Ar1a (Szeto et al., 2000a). Close inspection of the primary structure reveals that a number of charged amino acids at the N-terminus are not conserved between δ-ACTX-Hv1b and δ-ACTX-Hv1a/Ar1a such that the cationic residues Lys\(^4\) and Arg\(^5\) of δ-ACTX-Hv1a/Ar1a are substituted by Ser\(^4\) and Asp\(^5\) in δ-ACTX-Hv1b. In addition, Asn\(^6\) of δ-ACTX-Hv1a/Ar1a is substituted by Gly\(^6\) and the hydrophobic residue Tyr\(^{22}\) in δ-ACTX-Hv1a/Ar1a is replaced by Lys\(^{22}\). This lends support to the hypothesis that these residues, putatively involved in binding, are important for determining insect and/or mammalian selectivity.

Given that δ-atracotoxins target both mammalian and insect Na\(_v\) channels they have considerable potential as tools to aid in the investigation of structural requirements for anti-insect vs. anti-mammal activity. The residues that differ between δ-ACTX-Hv1b and δ-ACTX-Hv1a/Ar1a therefore may give us an unexpected insight into the residues that facilitate interaction of δ-atracotoxins with insect Na\(_v\) channels. This raises the possibility that manipulation of key residues may enable construction of a functional mirror-image of δ-ACTX-Hv1b, namely a δ-atracotoxin that binds to insect, but not vertebrate, voltage-gated sodium channels. Such an insect-specific δ-atracotoxin homologue might be a useful biopesticide. However, the structural basis for these selective interactions with insect vs. mammalian Na\(_v\) channels still awaits elucidation of the contact surfaces between the various toxins and their receptor binding sites. This may lead to the development of more efficacious and more selective insecticidal toxins capable of being employed in a recombinant baculovirus model or used to design non-peptide mimetics that could be used in foliar sprays.
12. Development of bioinsecticides using insect-selective sodium channel neurotoxins

The simplest way in which the selective insecticidal activity of spider neurotoxins can be utilized is via the development of a recombinant baculovirus. Insertion of the gene encoding for the toxin into non-essential parts of the baculovirus genome greatly enhances the efficacy of natural insect-specific baculoviruses, reducing the ‘time-to-kill’, and thus increasing the pesticidal potential of these viruses (Hughes et al., 1997; Thiem, 1997). The baculovirus strain of choice for gene insertion is the Autographa californica nuclear polyhedrosis virus (AcNPV) as it infects various important lepidopterous pest insects (Elazar et al., 2001). The first successful insect-selective toxin to be inserted and functionally expressed in the baculovirus was AahIT, a neurotoxin isolated from the venom of the scorpion Androctonus australis hector (Zlotkin et al., 1971). Since then, other toxins such as TxP-1 from a predatory straw itch mite, Pyemotes tritici (Tomalski and Miller, 1991; Popham et al., 1997), As II and Sh I from the sea anemones Anemonia sulcata and Stichodactyla helianthus respectively (Prikhod’ko et al., 1996; Prikhod'ko et al., 1998) and other scorpions (McCutchen et al., 1991; Stewart et al., 1991; Cory et al., 1994; Gershburg et al., 1998; Sun et al., 2002) have been inserted into the recombinant baculovirus delivery system with some success. Importantly, three spider toxins have also been used including µ-Aga IV, DTX9.2 and TalTX-1 from the hobo spider Tegenaria agrestis (Tomalski et al., 1988; Tomalski et al., 1989; Prihod’ko et al., 1996; Hughes et al., 1997; Prikhod'ko et al., 1998). Insertion of genes that encode toxins, as those described above, has been shown to cause dramatic improvements in insecticidal speed of certain viruses, causing lepidopteran larvae to die '50% sooner than those larvae infected with wild-type forms of the viruses.

There are however potential drawbacks from using scorpions, mite and sea anemone toxins, due to the numbers of disulfide bonds in their structure (reduced likelihood of correct folding) and in many cases their slightly larger size. Some authors have concluded that highly potent insecticidal neurotoxins could be ineffective when incorporated into a baculovirus because the toxin fails to be properly processed or fold properly in virus-infected insects (Tedford et al., 2004b). Importantly, insecticidal spider toxins, including µ-Aga IV, contain typically 35-40 residues and are smaller than all the other insecticidal toxins (e.g. AahIT is composed of 70 residues, Txp1 comprises 252 residues, and Sh-I has 48 residues). Also the majority of spider toxins are composed of the ICK motif (Norton and Pallaghy, 1998). This structure provides the toxins with high chemical stability and resistance to denaturation and proteolysis. The motif acts as a stable scaffold for the side chains of key residues that interact with the receptor site (Norton and Pallaghy, 1998). The presence of the ICK motif and smaller molecular size makes the spider toxins ideal candidates for incorporation into the baculovirus genome, increasing the
probability that the toxins fold correctly and display the precise spatial arrangement of key residues. Indeed, heterologous overexpression systems have found that many insecticidal spider toxins re-fold in a straightforward manner (Wang et al., 2000; Tedford et al., 2004a). Thus while there had been success with scorpion and sea anemone toxins, spider toxins might offer greater insecticidal efficacy especially if they are more active, although this remains to be investigated. In addition, spiders have the largest pharmacological reservoir of any venomous animals on earth. There are an estimated 100,000 species of spiders and each species contains ~100-200 peptides in their venom, which leads to an estimated total of approximately 10-20 million spider-venom polypeptides (Escoubas et al., 2006). Thus far, it appears that only 0.01% of the spider venom polypeptides have been identified (Tedford et al., 2004b). Nevertheless, currently there is a limited use of recombinant baculoviruses expressing insecticidal neurotoxins due to a lack of well-characterized toxins from which to select for incorporation into the baculoviral genome. Thus it is essential to isolate and identify insecticidal neurotoxins from a variety of venomous creatures.

Other approaches include transgenic approaches to incorporate the toxins in plants for the control of phytophagous pests. The surprising oral toxicity of $\omega$-ACTX-Hv1a, an insect-selective Ca$_v$ channel blocker (Fletcher et al., 1997b), has been recently reported following incorporation into the tobacco plant *Nicotiana tabacum*; indeed a thioredoxin–$\omega$-ACTX-Hv1a fusion protein was found to be insecticidal in *Helicoverpa armigera* and *Spodoptera littoralis* caterpillars by topical application (Khan et al., 2006). In support, the same toxin has previously been reported to be toxic by oral administration to the American lone star tick *Amblyomma americanum* (Mukherjee et al., 2006). Nevertheless, this approach has so far not been attempted with any of the spider toxins targeting the Na$_v$ channel. However, the insecticidal potency of scorpion toxins targeting the Na$_v$ channel has been shown to be enhanced by fusion with the snowdrop lectin (*Galanthus nivalis* agglutinin; GNA). The insect scorpion toxin ButaIT from the scorpion *Mesobuthus tamulus*, was fused to GNA and expressed in the yeast *Pichia pastoris*. The fusion protein was significantly more toxic than either component alone when fed to larvae of the tomato moth *Lacanobia oleracea* or the rice brown planthopper *Nilaparvata lugens* (Trung et al., 2006). In a separate study, *Brassica napus* plants expressing a chitinase for digestion of the insect gut membrane were also modified to co-express the scorpion insect toxin BmK IT from *Buthus occitanus* Karsh. The resultant transgenic plant was resistant to attack by phytophagous insect pests (Wang et al., 2005). Additional measure may also increase toxicity of recombinant baculoviruses or transgenic plants. Co-expression of two synergistic toxins has been shown to increase insecticidal potency (Regev et al., 2003) as has been noted with components within an individual arachnid venom
These findings open up a variety of molecular approaches for neurotoxin delivery beyond that using a simple recombinant baculoviral method.

13. Conclusions and future directions

Given that spiders rely upon their venom to immobilize or kill their prey it is not surprising that they contain a wide variety of insect-selective toxins that may provide the basis for the development of biopesticides. These neurotoxins often target specific insect prey or more precisely subtle differences in the prey’s nervous system. Interestingly a large number of these phyla-specific toxins target voltage-gated ion channels. These represent suitable targets for the future development of insecticides since they are ubiquitous amongst insects.

As pharmacologists we can take advantage of spider venom as a diversified source of molecular probes for identifying differences in phyla- and subtype-specificity of specific Na, channels and as leads for insecticides. Insect-selective spider toxins targeting the Na, channel have now been described and bind with each of the three major peptide toxin target sites: site-1 (HNTX-1), site-3 (Tx4(6-1) and Magi 2) and site-4 (δ-PaluIT2, μ-agatoxins and curtatoxins). These toxins thus validate sites 1, 3 and 4 on Na, channels as potential insecticide targets. Indeed μ-agatoxins have already been trialed in a recombinant baculovirus approach to insect control (Prikhod'ko et al., 1998). However, despite their significance and potential for application in insect-pest control, the structural basis for the selectivity of these toxins for insect over mammalian ion channels is still largely unknown. Thus spider toxins have considerable potential as tools to aid in the investigation of the molecular determinants for anti-insect versus anti-mammal activity as has been initiated with scorpion α-insect toxins (Karbat et al., 2004). The structural basis for these selective interactions requires elucidation of the contact surfaces (i.e. insectophore or pharmacophore) between the various toxins and their receptor binding sites on Na, channel subtypes. This may lead to the development of more efficacious and more selective insecticidal toxins capable of being employed in a recombinant baculovirus model, as fusion proteins with snowdrop lectins or used to design non-peptide mimetics that could be used in foliar sprays.

Small molecular weight spider toxins are proving valuable molecular probes for the investigation of the molecular topology of ion channels. Spider toxin probes can expose subtle phyla- or subtype differences at the receptor sites on the Na, channel. Toxins such as the δ-atracotoxins and δ-PaluIT toxins are highly potent and specific molecular tools that are assisting in defining common macrosites or ‘hot spots’, instead of the conventional independent structural sites, such as site-3 and -4,
previously defined using just scorpion $\alpha$- and $\beta$-toxins. In general the small size of spider toxins has distinct advantages over the larger scorpion toxins, including the ease of synthesis of alanine mutants to determine the bioactive surface of the molecule. Moreover, some spider toxins such as the $\delta$-PaluIT toxins will be useful tools to define novel links between sites modulating channel activation and inactivation given their unusual modulation of channel inactivation via an interaction with site-4.

The non-selective actions of several spider toxins illustrates the issue of cross-reactivity due to potentially conserved structures within domains of voltage-gated ion channels, particularly the voltage-sensor. However, target cross-reactivity also exemplifies a current issue with research to determine the target of spider neurotoxins. Many pharmacological studies investigate only a limited range of targets emphasizing the fact that these toxins may act with greater efficacy at other sites, and contribute to the overall physiological action of these agents, sometimes with a synergistic action in prey (eg. insect) species.

Presently, the number of spider toxins found to target the Na$_v$ channel is limited and, coupled with a lack of data on the 3D structures of these toxins, only a restricted number of folds are described. Indeed within the three groups of toxins targeting sites 1, 3 and 4, each group has only one type of 3D structure described. Nevertheless, other toxins with distinctive primary structures and unique disulfide bonding patterns have been described. Thus other folds are likely to be found within each of these groups, as has been determined for site-3 scorpion $\alpha$-toxins, sea anemone toxins and $\delta$-atracotoxins.

Screening of venom gland cDNA libraries from spider species can also provide important clues about the pharmacophore or insectophore of these toxins by comparison of the primary structure of toxin homologues. However, in isolation this approach has some drawbacks in that it does not provide any information about the biological activity of any novel homologues, especially insect vs. mammalian toxicity, and it also fails to identify posttranslational modifications critical for the activity of the mature toxin. Future structure-function studies to map the pharmacophores of these toxins using alanine-scan mutants or synthetic toxin analogues, combined with binding and electrophysiological approaches, should contribute to a more detailed mapping of spider neurotoxin binding to the Na$_v$ channel. This should provide structure-activity data critical for determining the phyla- or tissue-specific actions of spider toxins, but without precise identification of their binding site and mode of action their potential in the above areas remains underdeveloped.
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Table 1. Neurotoxin receptor sites 1-7 associated with the mammalian Na$_v$ channel indicating sites of putative spider toxin interaction.

<table>
<thead>
<tr>
<th>Neurotoxin receptor site</th>
<th>Neurotoxin(s)$^{a,b,c}$</th>
<th>Classical effects on ion permeation or gating</th>
<th>Allosteric coupling$^f$</th>
</tr>
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<tbody>
<tr>
<td>Site 1</td>
<td>Hainantoxin-I</td>
<td>Block ion conductance</td>
<td>+3, +5, −2</td>
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<tr>
<td></td>
<td>Hainantoxins III-V$^d$</td>
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<td>Huwentoxin-IV$^d$</td>
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<td>Tetrodotoxin (TTX)</td>
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<td>Saxitoxin (STX)</td>
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<td>μ-Conotoxin (μ-CTX)</td>
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<td>Batrachotoxin (BTX)</td>
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<td></td>
<td>Veratridine (VTN)</td>
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<tr>
<td>Site 2</td>
<td>Batrachotoxin (BTX)</td>
<td>Hyperpolarizing shift in voltage dependence of activation and inhibition of inactivation</td>
<td>+3, −6</td>
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<tr>
<td></td>
<td>Veratridine (VTN)</td>
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<tr>
<td></td>
<td>Aconitine</td>
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<td></td>
<td>Grayanotoxin (GTX)</td>
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<td></td>
<td>$N$-alkylamides</td>
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<tr>
<td>Site 3</td>
<td>δ-Atracotoxins (δ-CTX)</td>
<td>Hyperpolarizing shift in voltage dependence of activation</td>
<td>+2, −5</td>
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<td>δ-Missulenatoxin-Mb1a</td>
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<td></td>
<td>Magi 1 and Magi 4</td>
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<td></td>
<td>PnTx2-6</td>
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<tr>
<td></td>
<td>Jingzhaotoxin-I (JZTX-I)</td>
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<tr>
<td></td>
<td>Scorpion α-toxins</td>
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<td></td>
<td>Sea anemone toxins</td>
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<tr>
<td>Site 4</td>
<td>δ-Palutoxins (δ-PaluIT)$^d$</td>
<td>Strong hyperpolarizing shift in voltage dependence of activation, transient repetitive activity</td>
<td>+2, +4, −3</td>
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<td></td>
<td>μ-Agatoxins (μ-Aga)$^{d,e}$</td>
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<td></td>
<td>Curtatoxins$^e$</td>
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<td></td>
<td>Magi 5 toxin</td>
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<td></td>
<td>Scorpion β-toxins</td>
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<tr>
<td>Site 5</td>
<td>Brevetoxins (PbTx)</td>
<td>Hyperpolarizing shift in voltage dependence of activation and inhibit inactivation, repetitive activity</td>
<td>−2</td>
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<td>Ciguatoxins (CTX)</td>
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<tr>
<td>Site 6</td>
<td>δ-Conotoxins</td>
<td>Inhibit inactivation in molluscs</td>
<td>+2, +3, +5</td>
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<td></td>
<td></td>
<td>Inhibit inactivation, shift voltage dependence of activation and slow activation and deactivation kinetics, repetitive activity and/or block</td>
<td>+2</td>
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<tr>
<td>Site 7</td>
<td>Pyrethroids</td>
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<td></td>
<td>DDT and analogues</td>
<td></td>
<td></td>
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<tr>
<td>Unidentified site</td>
<td>Local anesthetics</td>
<td>Block ion conductance</td>
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<td></td>
<td>Anticonvulsants</td>
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<td>Dihydropyrazoles</td>
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</table>
Toxins in highlighted text are derived from spider venoms. aInsect-selective spider toxin(s) are shown in black highlighted text. bOther spider toxins are shown in grey highlighted text. cInsecticides are shown in italics. dBind to site 4 but slow insect Na\textsubscript{v} channel inactivation similarly to site-3 toxins. eThe inclusion of HNTXs and HWTX-IV as site-1 toxins and \(\mu\)-agatoxins and curtatoxins as site-4 toxins is speculative. fAllosteric coupling refers to the alteration in affinity of toxins binding at the mammalian neurotoxin receptor site (first column) by neurotoxin occupancy at the indicated site (final column). Positive cooperativity (+) indicates enhancement of binding of the toxin and/or stimulation of Na\textsuperscript{+} influx while negative cooperativity (–) refers to a decrease in toxin binding (Gordon et al., 1998). Table is adapted from (Cestèle and Catterall, 2000; Zlotkin et al., 2000; Nicholson et al., 2004).

**Figure Legends**

Fig. 1. Molecular structure and neurotoxin receptor sites of the Na\textsubscript{v} channel. (A) Schematic representation of the subunit structure of the Na\textsubscript{v} channel showing the functional \(\alpha\)-subunit (centre) comprising four homologous domains (I-IV) and ancillary \(\beta\)-subunits. Cylinders (S1-S6) represent putative transmembrane \(\alpha\)-helical segments within each domain where the charged S4 segments (red) represent the voltage sensors. The polypeptide chain is represented by the yellow ribbon and is approximately proportional to the length of the amino acid chain. The inactivation gate (magenta) is represented by the inactivation particle (hydrophobic residues IFM) with magenta arrows indicating the sites thought to form the inactivation gate receptor. The pore-lining segments S5 and S6, and intervening SS1/SS2 (P loop) that form the walls of the ion-conducting pathway are shown in blue. The extracellular domains of the \(\beta\)1 and \(\beta\)2 subunits are shown and represented as immunoglobulin-like folds similar to myelin protein P\textsubscript{0} (Shapiro et al., 1996). \(\Psi\), Sites of probable N-linked glycosylation. (B) Location of known neurotoxin receptor sites on Na\textsubscript{v} channels. Green circles represent the outer (EEDD) and inner (DEKA) rings of amino acid residues that form the ion selectivity filter and the proposed neurotoxin receptor site-1 for the water-soluble guanidinium toxins, tetrodotoxin (TTX) and saxitoxin (STX). Some \(\mu\)-conotoxin binding sites practically overlap with those of TTX and are omitted for clarity. In the case of receptor sites 3 and 4, only areas where there is more than a five-fold increase in binding affinity are highlighted. Insect-selective spider toxins are highlighted in red text. Figure is adapted from (Cestèle and Catterall, 2000)
Fig. 2. Evolution of DDH-related folds (Aa, Ab) into the ICK structural motif (Ba, Bb). Left hand columns in each panel (Aa, Ba) show schematic representations of the structural motifs depicting the formation of the cystine-knot and possible addition of the third $\beta$-sheet. $\beta$-Sheets are shown as grey arrows and disulfide bridges connecting cysteine residues are shown as dark grey lines with roman numerals. The dark arrow ($\beta$1) in panels Ba and Bb represents the additional $\beta$-sheet not always present in ICK spider toxins. Right hand columns in each panel (Ab, Bb) show a schematic view of the 3D structures of typical representatives of the two structural motifs. (Ab) HWTX-II (PDB 1I25) and (Bb) $\delta$-PaluIT2 (PDB 1V91). (C) Stereo view of the cystine knot of SGTx1 (PDB 1LA4).

Fig. 3. Comparison of the amino acid sequences of insect-selective toxins shown to target Na$_v$ channels. Identical residues are boxed in grey and conservatively substituted residues are in grey italic text. Where determined, disulfide-bonding patterns for the cysteine residues are indicated above the sequences.

Fig. 4. Structural comparison of spider toxins thought to target site-1 on the Na$_v$ channel. Schematic view of NMR solution structures of hainantoxin-I (PDB code 1NIX), hainantoxin-IV (PDB code 1NIY), and huwentoxin-IV (PDB code 1MB6) showing the location of $\beta$-strands (cyan) and helices (green and yellow). Disulfides bridges are shown as red tubes. In all of all these structures the disulfide bridges form an inhibitor cystine-knot (ICK) motif. The right-hand panels show surface representations of the toxins highlighting the putative pharmacophore of hainantoxin-IV (Li et al., 2004) and the topologically related residues in hainantoxin-I and huwentoxin-IV. Residues are coded: blue, positively charged; red, negatively charged or polar; magenta, aliphatic or aromatic. Toxin models were prepared using MOLMOL and PyMOL.

Fig. 5. Structural comparison of spider toxins targeting sites 3 and 4 on the Na$_v$ channel. Schematic view of NMR solution structures showing the location of $\beta$-strands (cyan) and helices (green and yellow). Disulfides bridges are shown as red tubes. (Aa) The left-hand panel shows the NMR solution structure of $\delta$-PaluIT2 (PDB file 1V91), known to interact with receptor site-4. The central panel highlights the side-chains of the known pharmacophore of $\delta$-PaluIT2 (Corzo et al., 2005). The right-hand panel shows the structure of Bj-xtrIT (PDB file 1BCG) (Cohen et al., 2004). Residues that form the bioactive surface of Bj-xtrIT are highlighted; note the topological similarity to residues in the
pharmacophore of δ-PaluIT2. (Ab) NMR solution structures of the structurally related toxins μ-Aga I (PDB file 1EIT) and the cis conformation of ACTX-Hi:OB4219 (PDB file 1KQH). Cis/trans isomerism of ACTX-Hi:OB4219 occurs at the bond preceding Pro\textsuperscript{30} (dark blue tubes). (B) NMR solution structures of δ-ACTX-Ar1a (PDB file 1VTX) and δ-ACTX-Hv1a (PDB file 1QDP), which are known to interact with neurotoxin receptor site-3. Surface representations are shown at the bottom of the panel indicating the putative pharmacophore of δ-ACTX-Hv1a and its similarity to residues in the known bioactive surface of LqhαIT (PDB file 1LQH). Residues are coded: blue, positively charged; red, negatively charged; yellow, polar uncharged; magenta, aromatic hydrophobe; green, aliphatic hydrophobe. All the spider toxins shown here contain an inhibitor cystine-knot (ICK) motif. Toxin models were prepared using MOLMOL and PyMOL.