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Spider Neurotoxins Targeting Voltage-gated Sodium Channels

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ABSTRACT

The voltage-gated sodium (Na_v) 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 sodium channel gating and / or kinetics. A number of polypeptide toxins from the venoms of araneomorph and mygalomorph spiders have been isolated and characterized that interact with several of these sites. Certain huwentoxins and hainantoxins appear to target site 1 to block Na_v channel conductance. The δ-atracotoxins and Magi 4 slow Na_v channel inactivation via an interaction with neurotoxin 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 the voltage-dependence of activation, an action analogous to scorpion β-toxins, but with both δ-palutoxins and μ-agatoxins slowing Na_v channel inactivation, a site 3 like action. Many spider toxins target undefined sites, while others are likely to cross-react with other ion channels due to conserved

structures within domains of voltage-gated ion channels. It is already clear, however, that many spider toxins represent highly potent and specific molecular tools to define novel links between sites modulating channel activation and inactivation. Other spider toxins show phyla-specificity and are being considered as lead compounds for the development of biopesticides. Others display tissue specificity via interactions with specific Na_v channel subtypes and should provide useful tools to delineate the molecular determinants to target ligands to these channel subtypes. 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, δ-atracotoxins, Magi toxins, δ-palutoxins, voltage-gated sodium channel, hainantoxins

I. INTRODUCTION

A. Sodium channel structure and function

The molecular properties of voltage-gated ion channels began some 25 years ago with the discovery of the voltage-gated sodium (Na_v) channel protein using high specificity and affinity neurotoxin labeling methods (Beneski and Catterall, 1980). Na_v channels are responsible for the initiation and propagation of the action potential in excitable cells. Their structure comprises principally of a poreforming ~2000-residue glycoprotein α-subunit in eukaryotic Na_v channels. The α-subunit is composed of four homologous but non-identical domains (I–IV) connected by cytoplasmic linkers (Fig. 1A). 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 reentrant loops (SS1-SS2) that dip into the transmembrane region of the protein (Li et al., 2001) (Fig. 1A). The S4 segments are positively charged and serve as voltage sensors to initiate voltage-dependent activation by moving outward under the influence of changes in the electric field (Yang et al., 1996). Sodium channel inactivation is mediated by a short intracellular loop connecting domains III and IV, containing the key residues IFM (West et al., 1992) (Fig. 1A). The α-subunit is also associated with auxiliary subunits (β1, β2, β3 and / or β4) that are required for normal kinetics and voltage-dependence of gating but are not required for ion flux (Schreibmayer et al., 1994; Isom et al., 1995; Yu et al., 2003) (Fig. 1A).

B. Neurotoxin receptor sites: orphan receptors for molecular probes

The Na_v channel is the primary molecular target of numerous therapeutic drugs (e.g. local anesthetics, anticonvulsants and antiarrhythmics) and insecticides (eg. pyrethroids). 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 seven identified neurotoxin binding sites, referred to as neurotoxin receptor sites 17 (Table 1 and Fig. 1B; for recent reviews see (Gordon, 1997b; 1997a; Cestèle and Catterall, 2000; Tan et al., 2005). Substances 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 sites that bind peptide toxins exist and, according to their functional characteristics on Na_v channels, these toxins can be classified as inducing either a depressant or excitatory phenotype. Site 1, located on the extracellular surface of the pore, binds the peptide μ-conotoxins as well as the guanidinium alkaloids tetrodotoxin (TTX) and saxitoxin (STX) (Fig. 1B). These toxins physically occlude the conduction pathway resulting in a depressant phenotype. Site 3 toxins, including the classical scorpion α-toxins, and some sea anemone toxins, bind to the S3-S4 extracellular loop in domain IV as well as unidentified residues in S5-S6 linkers in domains I and IV (Fig. 1B) and slow channel inactivation by trapping the S4 segment in the inward position (Cestèle et al., 1998). This site has also been shown to have complex allosteric interactions with site 2, shown to bind 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) (Table 1). Site 4 toxins include the scorpion β-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 (Cestèle et al., 1998) (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 β-toxins) or inhibition of the transition to the fast-inactivated state of the channel (eg. scorpion α-toxins). Finally, site 6 binds the δ-conotoxin TxVIA which slows channel inactivation but shows different allosteric modulation to site 3 (Table 1) (Cestèle and Catterall, 2000).

C. Sodium channel subtypes

To date, nine mammalian ($Na_v1.1$-1.9) and three invertebrate Na_v channel α-subunits (eg. *Drosophila para* gene) have been cloned, functionally expressed and characterized (Ramaswami and Tanouye, 1989; Goldin et al., 2000). While they share >75% homology and similar pharmacology, their responses to a variety of neurotoxins can be diverse. With the use of tetrodotoxin (TTX), two main categories of sodium channels have been characterized in mammals: the TTX-sensitive channels found essentially in mammalian brain and skeletal muscle ($Na_v1.1$-1.3, 1.4, 1.6 and 1.7), and the TTX-

resistant channels found in heart ($Na_v1.5$) and sensory neurons in peripheral ganglia ($Na_v1.8$ and 1.9). 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 (Wicher et al., 2001). Nevertheless, there are often subtle phyla-specific differences revealed by the use of neurotoxins, particularly those derived from scorpion venoms (Gordon, 1997a) and insect Na_v channels are often more sensitive to the actions of these neurotoxins (Warmke et al., 1997). Thus additional blockers or modulators are required to probe the physiological roles of these different subtypes of Na_v channels, particularly those involved in nociception, and to provide lead compounds for the development of insecticides and therapeutics or the validation of their targets.

D. Spider venoms: combinatorial peptide libraries

Spiders have developed a combinatorial peptide library in their venom, which they rely upon to kill or paralyze their prey. Thus their venoms contain potent neurotoxins and other bioactive compounds, many of which are selectively insecticidal or lethal to vertebrates including humans and specifically target ion channels (for a review of other spider toxins see this volume and (Escoubas et al., 2000b; Rash and Hodgson, 2002). In particular, some toxins, such as ω-agatoxin IVA and α-latrotoxin, have been invaluable molecular tools for the identification and characterization of ion channel subtypes and the process of neurotransmitter exocytosis, respectively (for reviews see (Adams, 2004; Ushkaryov et al., 2004). Others are now being investigated for their possible use as bioinsecticidal agents for the control of phytophagous pests or vectors of new or re-emerging disease (for a review of insecticidal spider neurotoxins see (King et al., 2002)).

The focus of this review is the spider toxins interacting with the Na_v channel. The review will detail the site and mechanism of action of these neurotoxins and discuss structure-function with relevance to the application of these neurotoxins in the fields of pharmacology and neuroscience and the development of novel insecticides. Omitted from this review are toxins interacting with proton-gated Na^+ channels (ASIC channels) such as psalmotoxin 1 from the venom of *Psalmopoeus cambridgei* (Escoubas et al., 2000a).

II. NA_V CHANNELS AS MOLECULAR TARGETS OF SPIDER TOXINS

A. Pore blocking toxins: potential site 1 ligands

Huwentoxin-IV and Hainantoxins I and III-V

Several toxins isolated from the venom of the Chinese bird spider *Ornithoctonus* spp. (formerly *Selenocosmia*) (Mygalomorphae: Theraphosidae) have been found to target the Na_v channel. Huwentoxin-IV (HWTX-IV) is a 35-residue polypeptide with three disulphide bridges (Fig. 2A) isolated from the venom of *Ornithoctonus huwena*. HWTX-IV is also similar in structure and function

to hainantoxins (HNTX) I and III-V from *Ornithoctonus hainana*. These latter toxins are composed of 33-35 residues with three disulphide bonds (Fig. 2A). 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 voltage-gated calcium (Ca_v) 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). Interestingly, an ortholog, HNTX-I, also has a similar action but displays a 15-fold increase in selectivity for the *para/tipE* insect Na_v channel than the rat $Na_v1.2/\beta1$ channel, with no effect on a variety of other Na_v channels (Li et al., 2003). 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 [^{3}H]-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 which 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 this novel site on the Na_v channel.

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

The hainantoxins and huwentoxin-IV contain a 'disulfide-knot' which places them in a class of toxins and inhibitory polypeptides with an 'inhibitor cystine-knot' (ICK) motif (Norton and Pallaghy, 1998). This is similar to marine molluscs and, importantly, all spider toxins targeting the Na_v channel whose disulfide-bonding pattern have been determined to date. However within this fold-class, the biological activities of other ICK toxins are quite diverse with activity at Na_v, Ca_v and voltage-gated potassium (K_v) channels, mechanosensitive channels, nicotinic acetylcholine receptors or ryanodine receptors. This highlights that different biological functions are often grafted onto the same or similar structural scaffolds. 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.

B. Excitatory spider toxins interacting with neurotoxin site 3: gating modifiers of inactivation

δ-*Atracotoxins*

Australian funnel-web spiders (Mygalomorphae: Hexathelidae: Atracinae) are a diverse group that is classified into two genera, *Atrax* and *Hadronyche* although all spiders in this sub-family are thought to have venom potentially toxic to primates (Torda et al., 1980; Graudins et al., 2002). A variety of peptides, known as atracotoxins (ACTXs), have been isolated from the venom of funnel-web spiders. However, the lethal toxins, responsible for the major primatespecific symptoms of envenomation, the δ-ACTXs have been shown to target the Na_v channel (for a complete review see (Nicholson et al., 2004)). A single primate-specific variant of the δ-ACTX family is the major component of most Australian funnelweb spider venoms including δ-ACTX-Hv1a from *Hadronyche versuta* (Sheumack et al., 1985), δACTXAr1a from *Atrax robustus* (Brown et al., 1988) and δ-ACTX-Hs20.1a from *Hadronyche* sp. 20 (Nicholson et al., 2004) (Fig. 2B), but some species produce multiple δACTX-1 homologues (Szeto et al., 2000). These δ-ACTXs are composed of 42 residues containing eight cysteines. The three-dimensional solution structures of δACTXHv1a (Fletcher et al., 1997) and δACTXAr1a (Pallaghy et al., 1997) have been determined using NMR spectroscopy (Fig. 4B) and consist of a triple-stranded antiparallel β-sheet, in some cases ending in a 3_{10} helix (eg. δ-ACTX-Hv1a).

Electrophysiological studies have identified that δ-ACTXs alter neuronal excitability in both insect and mammalian neurons by causing a prolongation of action potential duration. This results in the appearance of plateau potentials, accompanied by spontaneous repetitive firing (Grolleau et al., 2001; Alewood et al., 2003). δ-Atracotoxins were shown to produce a selective slowing of TTX-sensitive I_{Na} inactivation and a reduction in peak I_{Na}. In addition, the toxins caused a modest hyperpolarizing shift in the voltage dependence of activation (Nicholson et al., 1994; Nicholson et al., 1998; Szeto et al., 2000). These actions indicate that δ-ACTXs most likely interact with site 3 to inhibit conversion of the sodium channel from the open to the inactivated state, resulting in sodium current remaining at membrane potentials where inactivation is normally complete.

Using both $^{22}Na^+$ uptake and radiolabelled neurotoxin binding assays, it was found that δACTXs completely inhibit the binding of the classical scorpion αtoxins to site 3 in rat brain synaptosomes (Little et al., 1998a; Little et al., 1998b). Voltage-dependent binding to site 3 has recently been confirmed using ^{125}I-labelled δACTXHv1a and binding was inhibited competitively by the classical scorpion α-toxin Lqh-II and allosterically inhibited by brevetoxin1 in a manner similar to other site 3 toxins (Table 1) (Gilles et al., 2002). In light of the subtle variations in action and binding properties (Little et al., 1998b; Nicholson et al., 1998), it suggests that δACTXs interact at a non-identical yet overlapping site to that of scorpion α-toxins at site 3.

Interestingly δ-ACTXs are, in addition to being mammalian toxic, insecticidal showing similar signs of delayed contractile paralysis as the anti-insect scorpion αinsect toxin LqhαIT (Little et al., 1998a). In contrast to the phylaspecific actions of scorpion α-toxins, radioligand binding experiments revealed that δACTXs inhibit ^{125}I-labelled LqhαIT binding to cockroach neuronal membranes (Little et al., 1998a; Gilles et al., 2002). Therefore δACTXs 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., 2002). Notably, however, δACTXHv1a exhibits a low binding affinity to locust sodium channels (Gilles et al., 2002). Thus unlike scorpion toxins, which are only capable of differentiating between mammals and insects, δACTXs differentiate between insect Na_v channels from different insect orders (i.e. Dictyoptera vs. Orthoptera). Thus the actions of δ-ACTX suggest structural differences at the binding site between Na_v channels from the two insect orders.

Unfortunately, the structurefunction relationships of these toxins have not been determined directly. Importantly they do not share sequence homology or even fold homology with scorpion α-toxins such as Aah-II or sea anemone toxins. Nevertheless, attempts have been made to indirectly identify critical residues involved in δACTX 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 residues provide a complementary surface to the residues identified in the S3S4 loop of domain IV (Fletcher et al., 1997) or key residues forming the likely pharmacophores of scorpion toxins and sea anemone toxins (Gilles et al., 2002). These comprise of the residues highlighted in Figure 4B. Additional comparisons with the homologue δACTXHv1b, that lacks insecticidal activity (Szeto et al., 2000), reveals that a number of charged amino acids at the Nterminus are not conserved between δACTXHv1b and δACTXHv1a/Ar1a (Fig. 1B). This lends further support to the hypothesis that these residues, putatively involved in binding, are important for determining insect and/or mammalian selectivity.

δ-*Missulenatoxin*

A 42-residue neurotoxin, δ-missulenatoxin-Mb1a (δ-MSTX-Mb1a) sharing considerable sequence homology to the δ-ACTXs (Fig. 2B) has recently been described from the venom of the unrelated Australian eastern mouse spider *Missulena bradleyi* (Mygalomorphae: Actinopodidae). This toxin has been shown to slow TTX-sensitive, but not TTX-resistant, Na_v channel inactivation in rat DRG neurons in a manner almost identical to δ-ACTX toxins. However the toxin was approximately two-fold less toxic to insects than δ-ACTX-Hv1a (Gunning et al., 2003). Therefore, given the sequence homology and almost identical mode of action to δ-ACTXs, it most likely targets site 3 on the Na_v channel.

Magi toxins: Magi 1, 2 and 4

Six peptide toxins have been isolated from the Japanese funnel-web spider *Macrothele gigas* (Mygalomorphae: Hexathelidae) that display toxicity to mammals or insects. Of these five have been shown to displace radiolabelled neurotoxin binding to the Na_v channel. Magi 4, like the δ-ACTXs, is toxic to both insects and mammals and shares significant sequence homology with δ-ACTXs and δ-MSTX-Mb1a (Fig. 2B). Similarly it displaces LqhαIT binding to site 3 on insects, although it differs from the δ-ACTXs in that it fails to displace Lqh-II binding to rat brain synaptosomes (Corzo et al., 2003). Nevertheless, the authors have recently determined that Magi 4 slows Na_v channel inactivation of rat DRG neurons (unpublished observations) in a fashion analogous to the δ-ACTX family (Nicholson et al., 1994; Nicholson et al., 1998; Gunning et al., 2003).

Another toxin, Magi 2, has been found to induce flaccid 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. 2B). Magi 2 has been shown to displace the insect site 3 ligand ^{125}I-LqhαIT from cockroach synaptosomes with a K_i of 21 nM, whereas the K_i for displacement by Magi 1 is around 83-fold higher. They also fail to inhibit binding of radiolabelled neurotoxins to site 4 on insect, or site 3, 4 or 6 on mammalian Na_v channels. Comparison of the sequence of these two toxins may assist in the future identification of the pharmacophore given the distinct low toxicity of Magi 1.

Phoneutria Tx2 and Tx4 toxins

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). Fraction PhTx2, previously shown to cause the predominant effects of the whole venom (Araújo et al., 1993), has been purified and a 48-residue toxin, PnTx2-6, has been found to target the Na_v channel (Matavel et al., 2002). This toxin has five disulfide bonds but the 3D fold is yet to be determined. PnTx2-6 slows Na_v channel inactivation in frog skeletal muscle, reduces peak I_{Na} and induces a modest shift in the voltage dependence of channel inactivation. These actions are similar to those reported with δ-ACTXs and consistent with an interaction with site 3 on the Na_v channel. However PnTx2-6 displays only limited sequence homology with the δ-ACTXs, Magi 4 or δ-MSTX-Mb1a (Fig. 2B). Subtle differences between the actions of PnTx2-6 and other site 3 toxins on the recovery from inactivation and steady-state Na_v channel inactivation may also relate to differences in skeletal muscle and DRG neuronal Na_v channel subtypes.

Another novel toxin, Tx4(6-1), a 48-residue polypeptide with 5 disulfide bonds, has also been isolated from fraction PhTx4 (Figueiredo et al., 1995) (Fig. 2B). 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_v channel inactivation. This occurred in the absence of alterations to the voltage dependence of activation or steady-state inactivation. Patch clamp experiments on $Na_v1.2$ and $Na_v1.4$ channels expressed in *Xenopus oocytes* revealed that Tx4(6-1) failed to alter any aspects of Na_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 α-like toxin Bom IV for site 3 on insect Na_v channels (de Lima et al., 2002). Thus Tx4(6-1) together with Magi 2 represents a growing family of excitatory insect-selective spider neurotoxins targeting site 3 on the Na_v channel. Like other site 3 spider toxins it awaits fold and spatial structural determinations to delineate the molecular determinants of its specificity for the insect sodium channel.

Jingzhaotoxin I

Jingzhaotoxin I (JZTX-I) is a 33-residue polypeptide isolated from the venom of the Chinese tarantula *Chilobrachys jingzhao* (Mygalomorphae: Theraphosidae) (Xiao et al., 2004a). This toxin has three disulphide bonds and shows limited sequence homology to HNTX-I and HNTX-III (Fig. 2B). Despite this homology, JZTX-I has been shown to slow inactivation of TTX-sensitive Na_v channels of both rat DRG and insect neurons, albeit at high concentrations, as well as potent actions on TTX-resistant Na_v channels of rat cardiac myocytes. These actions occurred in the absence of changes in the voltage dependence of Na_v channel activation and indicate that JZTX-I most likely targets site 3 (Xiao et al., 2004a), although this awaits confirmation using competitive radioligand binding experiments. Interestingly, the toxin failed to modify TTX-resistant Na_v channels in rat DRG neurons but showed a 30-fold higher potency for inhibition of cardiac TTX-resistant I_{Na} than for insect or mammalian neuronal Na_v channels, thus discriminating cardiac from neuronal Na_v channel isoforms.

C. Excitatory spider toxins interacting with neurotoxin site 4: gating modifiers of activation

δ-*Palutoxins*

δ-Palutoxins (δ-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. 2C). 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 $Na_v1.2/β1$ channels at concentrations up to 10 μM (Ferrat et al., 2005). This action is similar to site 3 neurotoxins such as

the scorpion α-insect toxin, LqhαIT. Despite this they have been shown to displace the site 4 excitatory scorpion β-toxin, Bj-xtrIT, from binding on cockroach membranes and fail to displace LqhαIT binding (Corzo et al., 2005). In reciprocal experiments, Bj-xtrIT and the depressant scorpion β-toxin LqhIT2 also displaced ^{125}I-δ-PaluIT2 binding (Corzo et al., 2005). Thus δ-PaluITs represent the first spider toxins that definitively bind to site 4 on insect sodium channels but modulate Na_v channel inactivation. To date, only scorpion β-toxins have been shown to compete with this site. The NMR solution structures of the δ-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. δ-PaluIT1 and δ-PaluIT2 contain double and triple-stranded anti-parallel β-sheets, respectively. Alanine scanning mutagenesis experiments reveal that the putative insectophore of δ-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. 4Aa). 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.

μ-*Agatoxins*

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 close sequence homology to the curtatoxins from the related agelenid spider *Hololena curta* (Stapleton et al., 1990) (Figure 2C). 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_v 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 calcium entry into nerve terminals resulting in the increased frequency of miniature excitatory junctional potentials (mEJC). 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_v 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. 2C). The similarities in primary sequence and pharmacology of these toxins provides 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.

Magi 5 toxin

Magi 5, a novel 30-residue toxin from the venom of the spider *Macrothele gigas* (Fig. 2C), is also believed to interact with site 4 of the Na_v channel. It has been shown to be lethal to mice and produce a transient paralysis in insects (Corzo et al., 2003). Competitive binding assays have revealed that Magi 5 competes with a scorpion β-toxin active on mammals, ^{125}I-CssIV, for binding to site 4 on the Na_v channel. Interestingly, Magi 5 shows an intermediate potency to displace LqhαIT binding to insect site 3 on cockroach synaptosomes (Corzo et al., 2003). The precise mechanism of action therefore awaits analysis by electrophysiological methods.

D. Toxins interacting with unidentified sites on the Na_v channel

ACTX-Hi:OB4219: a novel atracotoxin awaiting target determination

Funnel-web spider venoms have been rich sources of toxins targeting Na_v channels, in particular the δ-ACTXs. Venom of the funnel-web spider *Hadronyche infensa* Orchid Beach also contains a 38-residue polypeptide, ACTX-Hi:OB4219, containing four disulphide bonds but with no significant homology to the δ-ACTXs. 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^{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. 4Aa-b), ACTX-Hi:OB4219 shares limited sequence identity with these toxins (Fig. 2C and D). Therefore, while ACTX-Hi:OB4219 may target Na_v channels, these differences in the primary sequence require electrophysiological or binding studies to definitively determine the target of ACTX-Hi:OB4219.

Jingzhaotoxin-III

Unlike JZTX-I, which appears to slow cardiac Na_v channel inactivation via an interaction with site 3, a related jingzhaotoxin (JZTX-III; Fig. 2D) significantly inhibited cardiac TTX-resistant I_{Na}

with no change in inactivation kinetics (Xiao et al., 2004b). This depressant spider toxin showed no effects on Na_v or Ca_v channels in DRG neurons or K_v channels expressed in *Xenopus oocytes*. Unlike scorpion β-toxins, it also caused a 10-mV depolarizing shift in the threshold of Na_v channel activation, reducing the probability of channel activation. Thus, JZTX-III is a novel toxin that, like JZTX-I, displays higher selectivity for cardiac Na_v isoforms acting via a novel mechanism. This spider toxin therefore represents a new ligand for discriminating the cardiac $Na_v1.5$ channel subtype.

Diguentia toxins

A family of 56-61 insecticidal polypeptides (DTX9.2, 10 and 11) have been isolated from the primitive weaving spider *Diguentia canities* (Araneomorphae: Diguentidae) (Krapcho et al., 1995; Bloomquist et al., 1996). DTX9.2 causes repetitive EPSP discharges in housefly larvae neuromuscular and sensory nerve preparations and a depolarization of cockroach axons, actions that were are blocked by TTX (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_v channels, but it is unlikely that DTX9.2 interacts with site 3.

E. Toxins with non-selective actions on voltage-gated ion channels: promiscuous toxins

It has been previously noted that peptide toxins can exert their actions both within and across voltage-gated ion channel families. For example the 41-residue polypeptide SNX482 (Fig. 2E) from the venom of the Cameroon red baboon tarantula *Hysterocrates gigas* (Mygalomorphae: Theraphosidae) is a known blocker of R-type (Newcomb et al., 1998) and P/Q-type (Arroyo et al., 2003) Ca_v channels. This toxin has also been shown to delay Na_v channel inactivation and partially block I_{Na} in bovine chromaffin cells at similar concentrations to those that block I_{Ca} (Arroyo et al., 2003). Such target cross-reactivity is also likely with Magi 3 from *Macrothele gigas*. This peptide 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-LqhαIT binding to cockroach synaptosomes (Corzo et al., 2003). The primary sequence of Magi 3 (Fig. 2E), 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 ω-agatoxin IVA which also decreased TTX-sensitive I_{Na} amplitude, enhanced I_{Na} decay and led to a slower recovery from Na_v channel inactivation in cockroach DUM neurons (Wicher and Penzlin, 1998). Finally Protoxin-I and -II (ProTx-I and -II) from the venom of the Brazilian green velvet

tarantula *Thrixopelma pruriens* (Mygalomorphae: Theraphosidae) (Fig. 2E) act as gating modifiers to inhibit $Na_v1.2$, 1.5, 1.7 and 1.8 channels by causing a depolarizing shift in the voltage-dependence of activation (Middleton et al., 2002). However ProTx-1 also potently blocks $Ca_v3.1$ (T-type) channels and partially inhibits $K_v1.3$ and $K_v2.1$ channels (Middleton et al., 2002) while ProTX-II blocks $Ca_v3.x$ (T-type) and $Ca_v1.x$ (L-type) Ca channels (Kraus et al., 2000).

This promiscuous activity of certain spider toxins on multiple voltage-gated ion channels may arise due to common structural elements shared between ion channels particularly Na_v and Ca_v. 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 hanatoxin and grammotoxin, two related protein toxins found in the venom of the Chilean rose tarantula (*Phrixotrichus spatulata*), both recognize a voltage-sensing domain of K_v and Ca_v channels (Li-Smerin and Swartz, 1998).

III. CONCLUSIONS AND FUTURE DIRECTIONS

Using several molecular mechanisms, spiders, like marine molluscs, have developed a combinatorial peptide library strategy to diversify their toxin pool (Sollod et al., 2005)naka. 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 the spider, enabling them to efficiently immobilize a wide variety of prey species. As pharmacologists we can take advantage of this pre-optimized peptide library as a diversified source of molecular probes for identifying differences in phyla- and subtype-specificity of specific Na_v channels and as leads for novel therapeutics and insecticides.

Given that spiders, particularly mygalomorphs, 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. However the insect Na_v channel, as opposed to other ion channels, presently represents the best long-term potential target for the development of novel insect-selective biopesticides and the least likely to develop resistance to candidate bioinsecticides. This arises because (i) it has been shown to possess a large number of binding sites, far more than other insecticidal toxins targeting Ca_v and K_v channels, thus 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 arachnid toxins, and (iii) its pharmacological diversity and flexibility as exhibited by the allosteric coupling of these binding sites (Zlotkin, 1999). Insect-

selective spider toxins targeting the Na_v channel have now been described and bind with each of the three major peptide toxin target sites: site 1 (HNTX-I), site 3 (Tx4(6-1) and Magi 2) and site 4 (δ-PaluIT2, μ-agatoxins and curtatoxins). These validate sites 1, 3 and 4 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 antiinsect versus antimammal 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 sodium 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 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_v 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 α- and β-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 δ-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 ability of spider toxins to discriminate between Na_v channel subtypes in mammalian tissues is of potential value for future rational design of novel therapeutics. JZTX-I and -III have been shown to selectively modulate cardiac $Na_v1.5$ channels and may prove useful tools in discriminating this channel from other TTX-resistant channels such as $Na_v1.8$ and $Na_v1.9$ important in pain perception. Both ProTx-I and ProTx-II were isolated on the basis of their ability to inhibit the TTX-resistant $Na_v1.8$ channel distributed in small diameter nociceptive DRG neurons in an attempt to find a novel analgesic compound. Unfortunately these toxins also inhibit other Na_v channels subtypes and Cav channels at similar concentrations. Therefore the hunt for a selective blocker of $Na_v1.8$ or 1.9 channels continues.

The non-selective actions of protoxins illustrate the issue of cross-reactivity due to potentially conserved structures within domains of voltage-gated ion channels, particularly the voltage-sensor.

Thus promiscuous toxins such as hanatoxin, grammotoxin and protoxins are likely to be useful ligands in determining the molecular basis of these conserved domains of ion channels. However, target cross-reactivity also exemplifies a current issue with research to determine the ion channel or receptor target of spider and other peptide 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 sequences 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 α-toxins, sea anemone toxins and δ-atracotoxins. The determination of the solution structure will be critical if the key residues on the bioactive surfaces are to be determined.

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 sequence of toxin homologues. However, in isolation this approach has some drawbacks in that it does not provide any information of biological activity of any novel homologues, especially insect vs. mammalian toxicity. 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 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.

Neurotoxin receptor site	Neurotoxin(s)	Classical effects on ion permeation or gating	Allosteric coupling
Site 1	**Hainantoxin-I** **Hainantoxins III-V** **Huwentoxin-IV** Tetrodotoxin (TTX) Saxitoxin (STX) μ-Conotoxin (μ-CTX)	Block ion conductance	+3, +5, –2
Site 2	Batrachotoxin (BTX) Veratridine (VTN) Aconitine Grayanotoxin (GTX) N-alkylamides	Persistent activation Hyperpolarising shift in voltage dependence of activation and inhibition of inactivation	+3, –6
Site 3	**δ-Atracotoxins (δ-ACTX)** **δ-Missulenatoxin-Mb1a** **Magi 1, 2** **Tx4(6-1)** **Jingzhaotoxin-I (JZTX-I)** Scorpion α-toxins Sea anemone toxins	Inhibit inactivation, minor hyperpolarising shift in voltage dependence of activation	+2, –5
Site 4	**δ-Palutoxins (** **μ-Agatoxins (** **Curtatoxins** **Magi 5 toxin** Scorpion β-toxins	Strong hyperpolarising shift in voltage dependence of activation, transient repetitive activity	
Site 5	Brevetoxins (PbTx) Ciguatoxins (CTX)	Hyperpolarising shift in voltage dependence of activation and inhibit inactivation, repetitive activity	+2, +4, –3
Site 6	δ-Conotoxins	Inhibit inactivation in molluscs	–2
Site 7	Pyrethroids DDT and analogues	Inhibit inactivation, shift voltage dependence of activation and slow activation and deactivation kinetics, repetitive activity and/or block	+2, +3, +5
Unidentified site	Local anaesthetics Anticonvulsants Dihydropyrazoles	Block ion conductance	+2

Toxins in bold text are derived from spider venoms. [a]Insect-selective toxin(s). [b]The inclusion of HNTXs and HWTX-IV as site 1 toxins and μ-agatoxins and curtatoxins as site 4 toxins is speculative. [c]Bind to site 4 but slow insect Na_v channel inactivation similarly to site 3 toxins. [d]Allosteric coupling

refers to the alteration in affinity of toxins binding at the 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^+ 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).

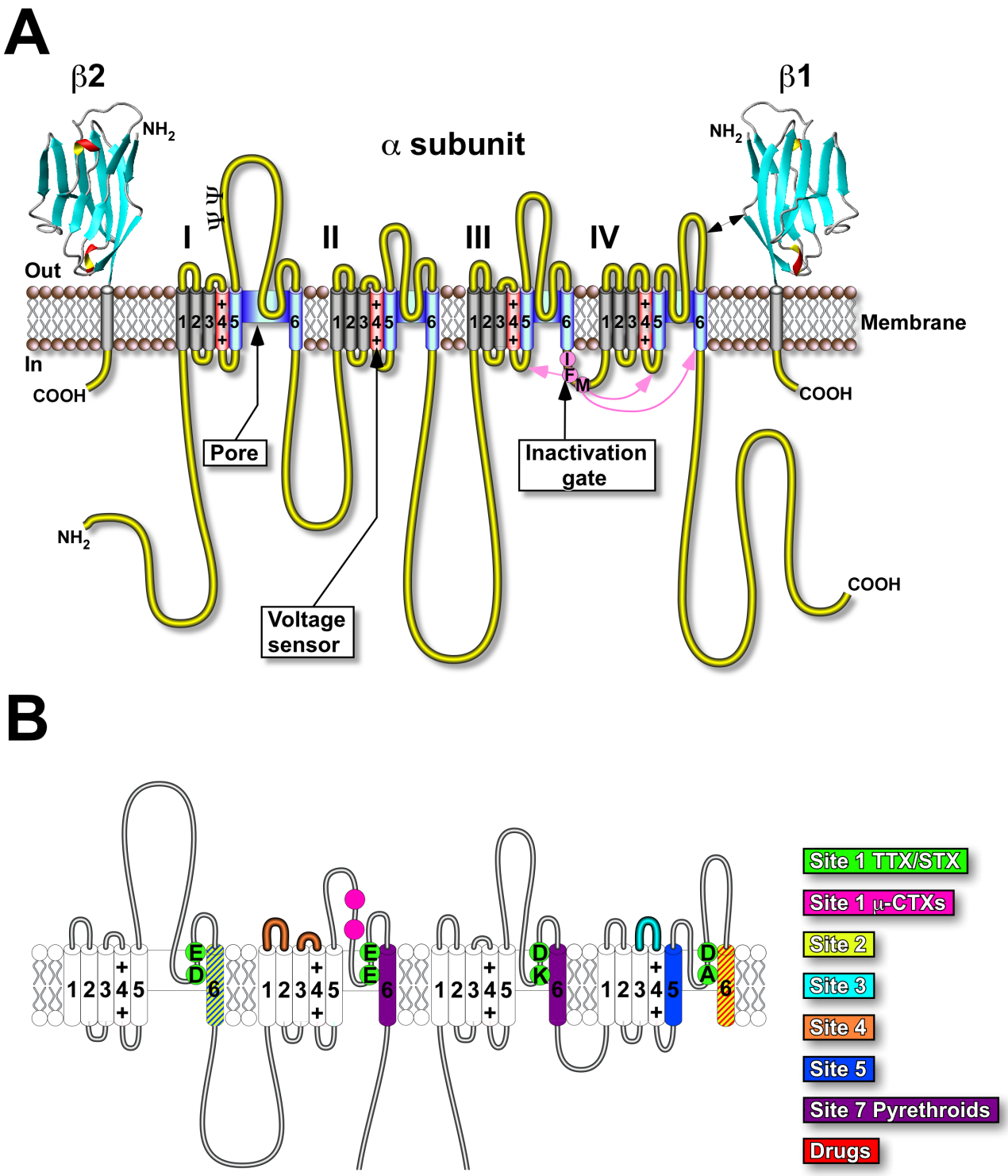
Figure Legends

Fig. 1. Molecular structure and neurotoxin receptor sites of the Na_v channel. (A) Schematic representation of the subunit structure of the Na_v channel showing the functional α-subunit (centre) comprising four homologous domains (I-IV) and ancillary β-subunits. Cylinders (S1-S6) represent putative transmembrane α-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 β1 and β2 subunits are shown and represented as immunoglobulin-like folds similar to myelin protein P_0 (Shapiro et al., 1996). Ψ, sites of probable N-linked glycosylation. (B) Location of known neurotoxin receptor sites on Na_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 of μ-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. Figure is adapted from (Cestèle and Catterall, 2000)

Fig. 2. Comparison of the amino acid sequences of 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. (A) Pore-blocking toxins on the Na_v channel. (B) Toxins targeting site 3 to slow inactivation. (C) Toxins targeting site 4. (D) Toxins interacting with unidentified sites on Na_v channels. Note: the disulfide bonding pattern of JZTX-III is the same as JZTX-I (see panel B). (E) Toxins with non-selective actions on Na_v and other voltage-gated ion channels. For comparison, Magi 3 is aligned with PLTXs that are known to block Ca_v channels.

Fig. 3. 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 β-strands (black arrows) and helices (light and dark grey helices). Disulfides bridges are shown as light grey tubes. In all structures disulfides bridges form an inhibitor cystine-knot (ICK) motif. Surface representations are shown to the right of the panel indicating the suspected pharmacophore of hainantoxin-IV (Li et al., 2004) and its similarity to topologically related residues in hainantoxin-I and huwentoxin-IV. Residues are coded: black, positively charged; light grey, negatively charged or polar; dark grey, aromatic. Toxin models were prepared using MOLMOL and PyMOL.

Fig. 4. 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 β-strands (black arrows) and helices (light and dark grey helices). Disulfides bridges are shown as light grey tubes. (Aa) NMR solution structures of δ-PaluIT2 (PDB file 1V91), known to interact with receptor site 4. Ribbon representations are shown in the middle and right of the panel indicating side-chains of the known pharmacophore of δ-PaluIT2 (Corzo et al., 2005) and its similarity to residues forming the known bioactive surface of Bj-xtrIT (PDB file 1BCG) (Cohen et al., 2004). (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^{30} (dark grey tubes). (B) NMR solution structures of δ-ACTX-Ar1a (PDB file 1VTX) and δ-ACTX-Hv1a (PDB file 1QDP) 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; Gilles et al., 2002). Residues are coded: black, positively charged; light grey, negatively charged or polar; dark grey, aliphatic or aromatic. In all the spider toxins disulfide bridges form an inhibitor cystine-knot (ICK) motif. Toxin models were prepared using MOLMOL and PyMOL.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In
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COOH
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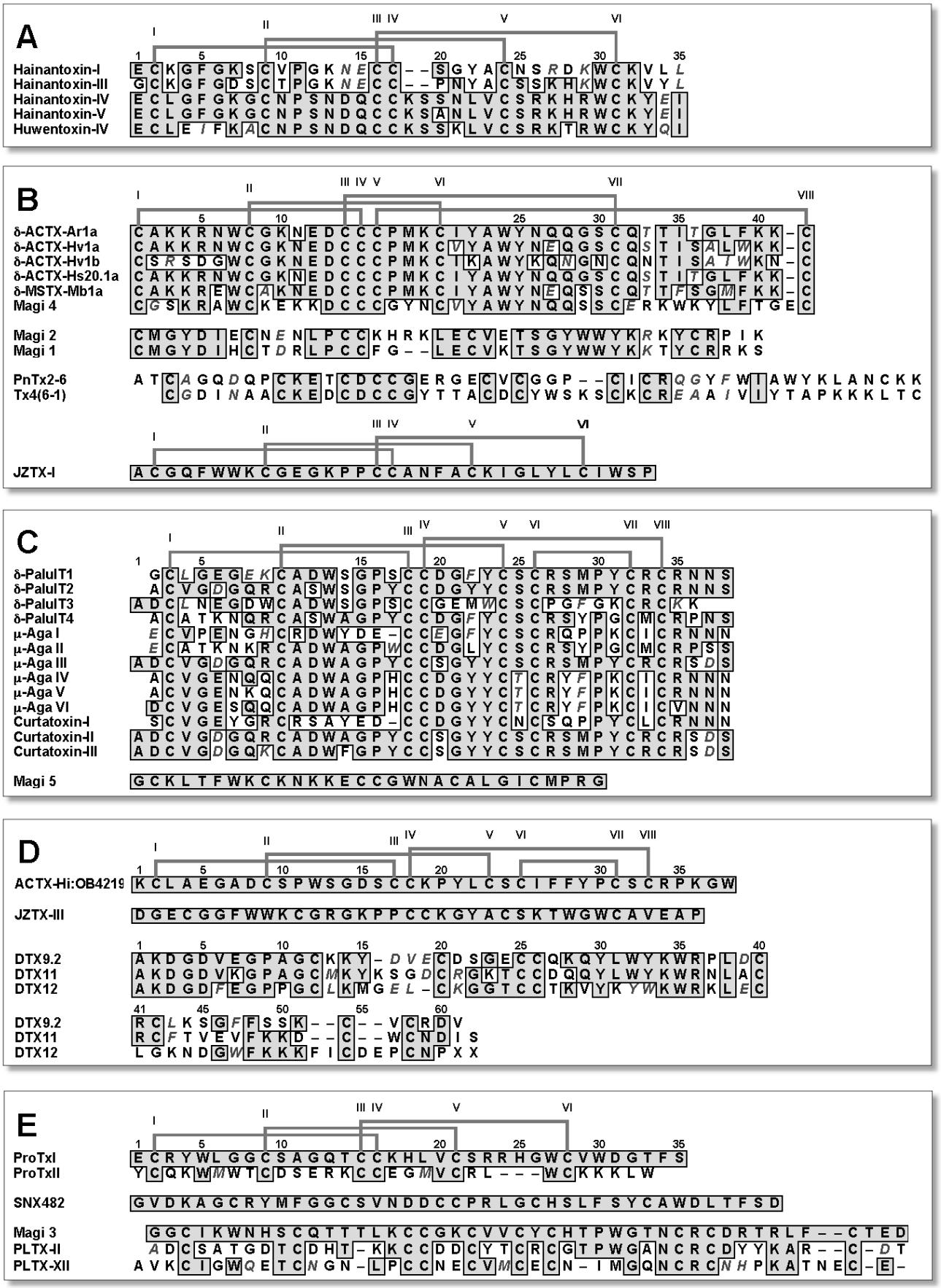
III
IV
Membrane
Pore
Voltage sensor
Inactivation gate
I F M
NH2
COOH
B
Site 1 TTX/STX
Site 1 μ-CTXs
Site 2
Site 3
Site 4
Site 5
Site 7 Pyrethroids
Drugs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Magi 2
Magi 1
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C
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μ-Aga III
μ-Aga IV
μ-Aga V
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Curtatoxin-II
Curtatoxin-III
Magi 5
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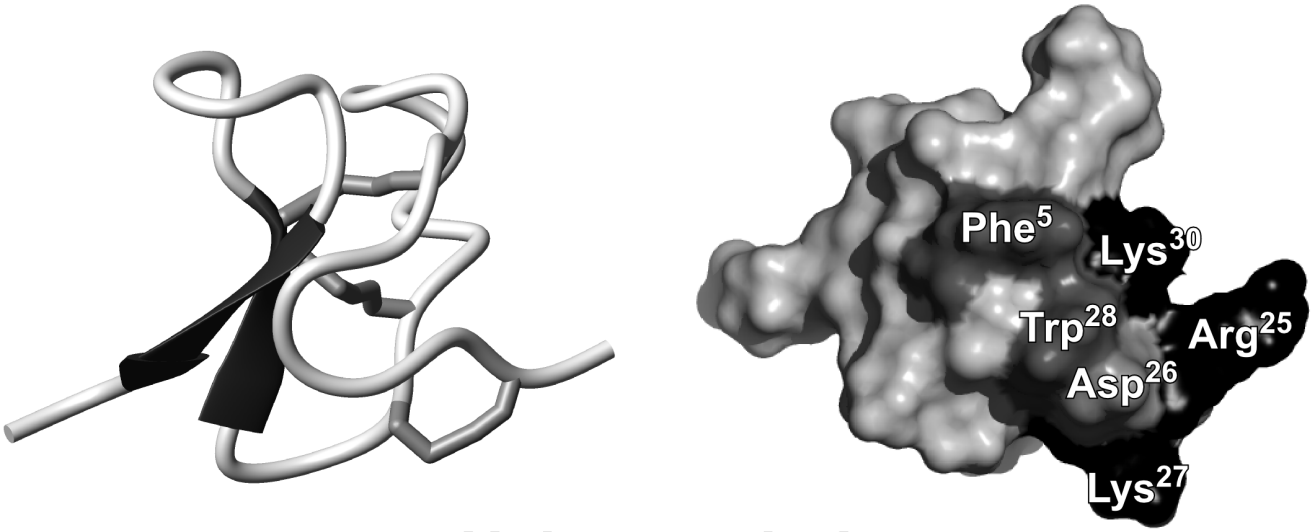
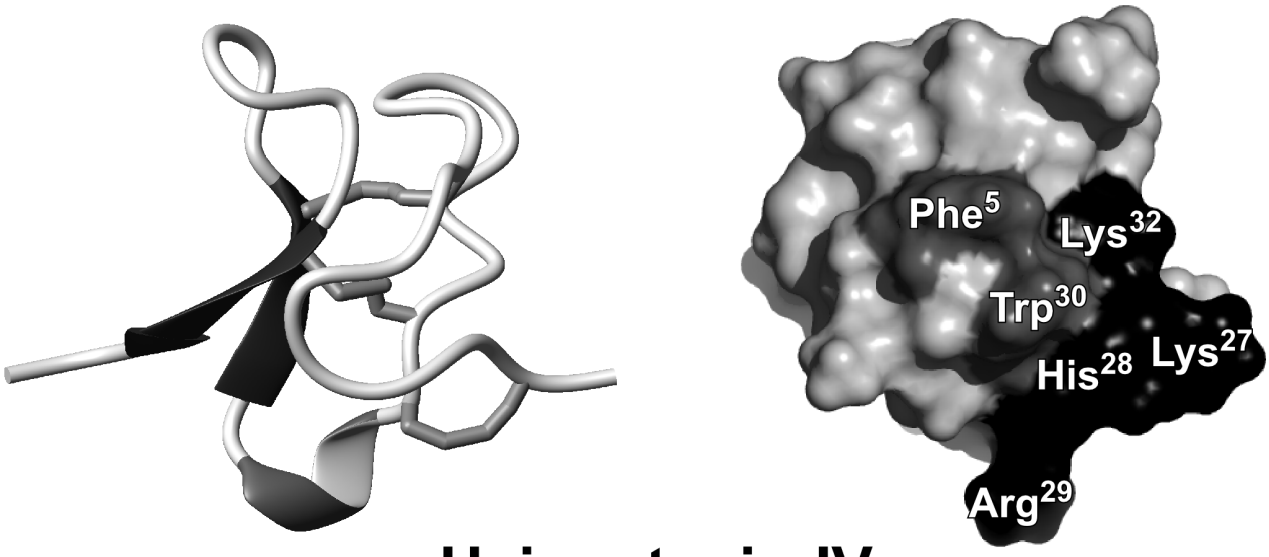


Hainantoxin-I



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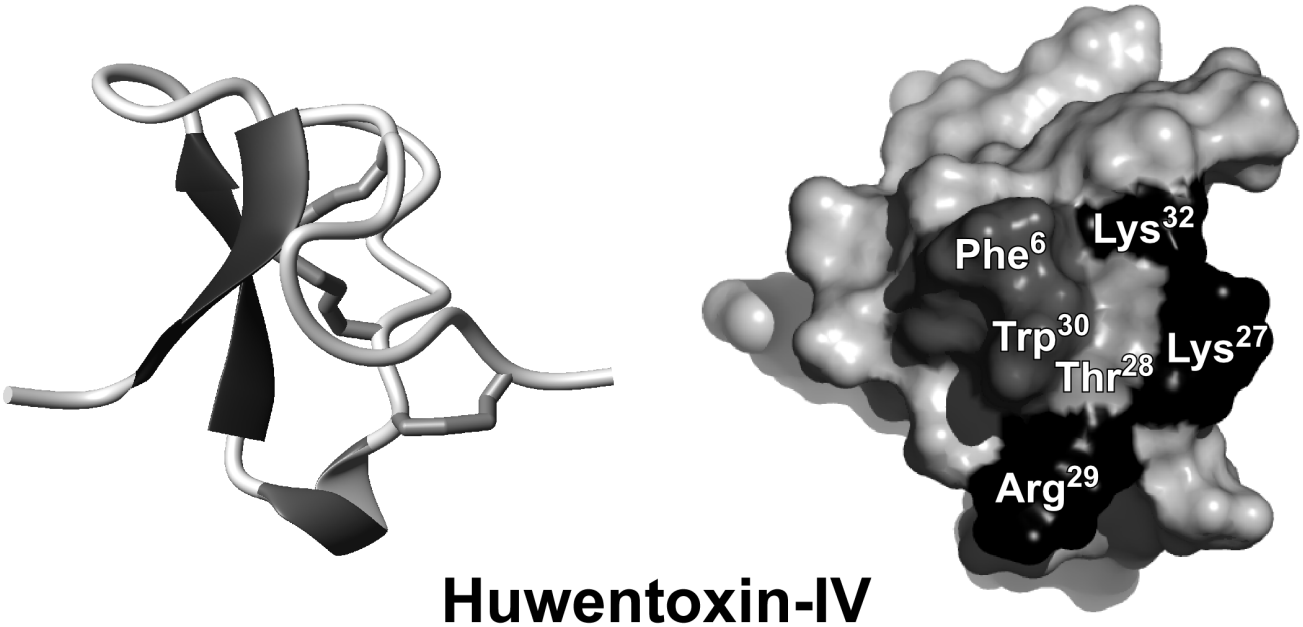
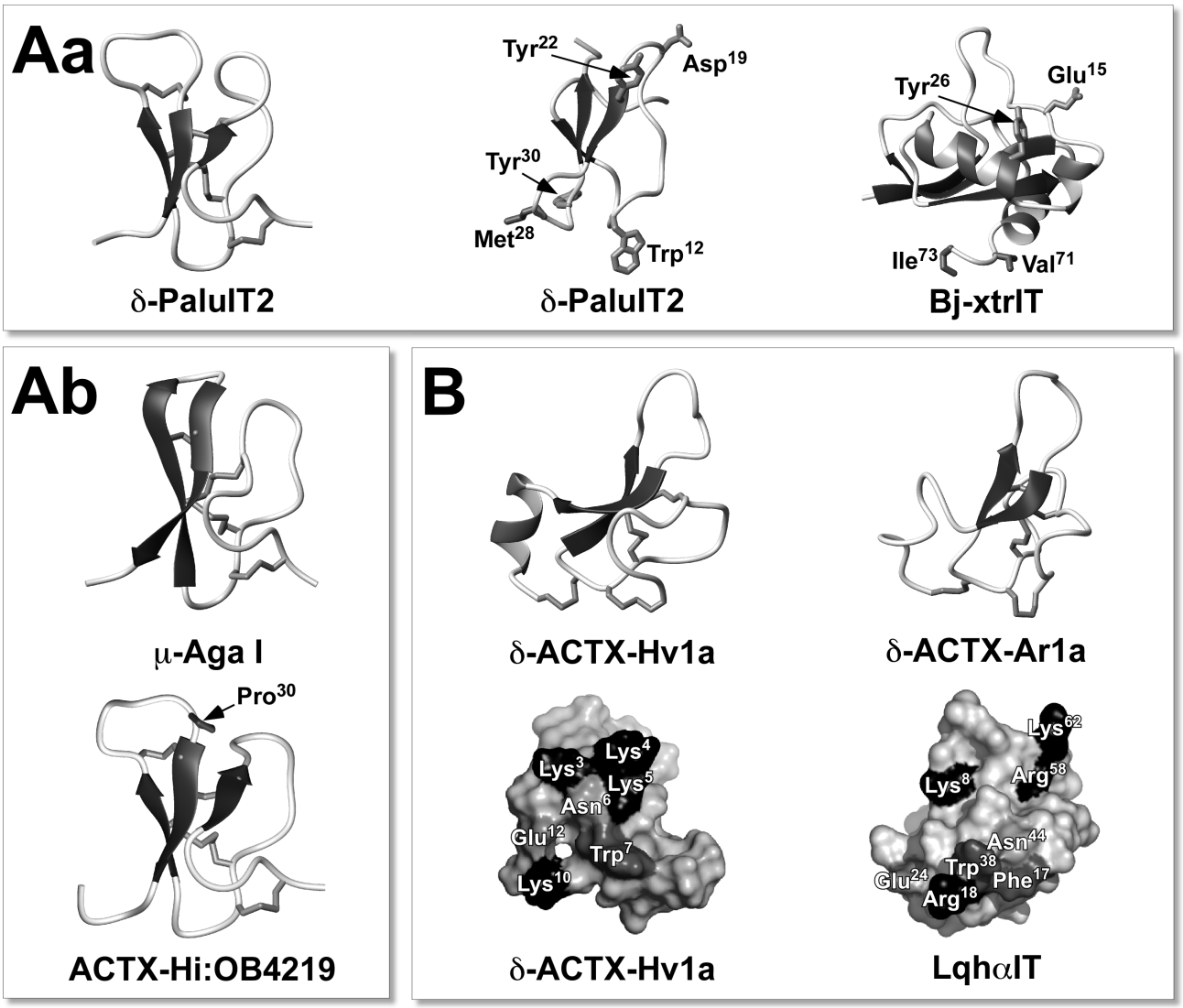


Huwentoxin-IV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Ab
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δ-ACTX-Ar1a
Lys4
Lys3
Lys5
Asn6
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