Snake three-finger α-neurotoxins and nicotinic acetylcholine receptors: molecules, mechanisms and medicine

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Abstract

Snake venom three-finger α-neurotoxins (α-3FNTx) act on postsynaptic nicotinic acetylcholine receptors (nAChRs) at the neuromuscular junction (NMJ) to produce skeletal muscle paralysis. The discovery of the archetypal α-bungarotoxin (α-BgTx), almost six decades ago, exponentially expanded our knowledge of membrane receptors and ion channels. This included the localisation, isolation and characterization of the first receptor (nAChR); and by extension, the pathophysiology and pharmacology of neuromuscular transmission and associated pathologies such as myasthenia gravis, as well as our understanding of the role of α-3FNTxs in snakebite envenomation leading to novel concepts of targeted treatment. Subsequent studies on a variety of animal venoms have yielded a plethora of novel toxins that have revolutionized molecular biomedicine and advanced drug discovery from bench to bedside. This review provides an overview of nAChRs and their subtypes, classification of α-3FNTxs and the challenges of typifying an increasing arsenal of structurally and functionally unique toxins, and the three-finger protein (3FP) fold in the context of the uPAR/Ly6/CD59/snake toxin superfamily. The pharmacology of snake α-3FNTxs including their mechanisms of neuromuscular blockade, variations in reversibility of nAChR interactions, specificity for nAChR subtypes or for distinct ligand-binding interfaces within a subtype and the role of α-3FNTxs in neurotoxic envenomation are also detailed. Lastly, a reconciliation of structure-function relationships between α-3FNTx and nAChRs, derived from historical mutational and biochemical studies and emerging atomic level structures of nAChR models in complex with α-3FNTxs is discussed.

Keywords

α-neurotoxins | snake three-finger toxin | snakebite envenomation | nicotinic acetylcholine receptor | cholinergic neurotransmission | protein structure-function

1 | Toxins from snake venom

Animal venoms comprise a cocktail of hundreds of toxins intended to incapacitate, kill and digest prey or deter predators [1, 2]. The selection, evolution, variation and production of this vast array of toxins have been moulded through the evolutionary predator-prey dichotomy over millennia (see reviews [3-9]). Despite their wide variability, animal toxins are limited to few structural superfamilies [10]; broadly categorised as *enzymatic proteins* including phospholipases A_2 (PLA_2), serine proteases, snake venom metalloproteases, acetylcholinesterases and phosphodiesterases (see review [11]), and *non-enzymatic proteins* that comprise three-finger toxins (3FTXs), serine proteinase inhibitors, C-type lectin-related proteins, disintegrins, helveprins, sarafotoxins, waprins, vespryns and natriuretic peptides (reviewed in [12]). While enzymatic toxins produce slow immobilization and digestion of prey, non-enzymatic toxins can swiftly shut down physiological processes by eliciting peripheral and central neurotoxicity, cytotoxicity, hemotoxicity and myotoxicity, driven by their ability to bind to their molecular targets with high affinity and precise selectivity [10-12].

Their toxicity notwithstanding, these molecules have made extraordinary contributions to scientific discovery as well as being a veritable source for drug leads [2, 13-16]. The 3FTx family, the most abundant of non-enzymatic proteins, have been described in Elapidae, Colubridae and Viperidae species [17-21]. Their typical protein fold, defined by three finger-like loops extending from a globular core, utilizes distinct pharmacophores to target a wide variety of physiological processes [17, 20]. This review will focus on α-3FNTxs that act on postsynaptic nAChRs to produce skeletal muscle paralysis [17-19, 22-24]. The prototypical α-3FNTx, α-BgTx, has been vital for the isolation and characterization of nAChRs, understanding the neurobiology of the NMJ and its

pathologies such as myasthenia gravis [25], and providing insight into snakebite envenomation neurotoxicity and the basis for emerging strategies for its treatment [26, 27].

2 | Historical perspectives of three-finger α-neurotoxin research milestones

While crude isolates of snake venom that produced neurotoxicity were obtained from cobras by Toyosaku Sasaki (1957), and from sea snakes by Carey and Wright (1960); Chang and Lee were the first to isolate and characterize three neurotoxins from the many-banded krait (*Bungarus multicinctus*) in 1963, using zone electrophoresis on starch [28]. The slowest moving fraction was named α-BgTx and shown to produce postsynaptic nAChR inhibition; whereas the faster moving fractions with presynaptic activity, were denoted with β and γ prefixes respectively. Interestingly, thereafter, any animal toxin from various phyla found to exhibit postsynaptic nAChR activity was given the 'α-' prefix and categorised as α-neurotoxins (see the story of α-BgTx [25]). The plant alkaloid d-tubocurarine, a competitive antagonist of muscle nAChRs that was introduced into clinical practice as a muscle relaxant in 1942 [29], was found to prevent α-BgTx binding, suggesting that α-BgTx occupied the same binding sites on the nAChR [30]. This set in motion the use of α-BgTx to advance the study of the cholinergic circuitry (see a historical perspective on the discovery of the nAChR [31]). Therein, α-neurotoxins also came to be known as "curaremimetic" toxins as they mimicked the pharmacological activity of d-tubocurarine [22, 24].

In the midst of several studies on the primary sequences and bioactivity of neurotoxins from cobras, kraits and sea snakes (see a reflection on the discovery of sea snake toxins [32]), Karlsson, Eaker and Porath first sequenced the amino acid residues of 'toxin α' from the spitting cobra (*Naja nigricollis*) in 1966 [33]. This paved the way for early structure-function studies in the 1970s, utilizing chemical modification [34], radioiodination [35], tritiation [36], monolabeling with fluorescent, spin or photoactivatable groups [37] and cross-linking of α-3FNTxs and nAChRs [38].

An alternative for venom purified α-3FNTxs was found in their heterologous expression in bacteria or yeast [39, 40], which facilitated seminal mutagenesis work by Menez and co-workers to decipher the pharmacophores of α-3FNTxs [41, 42]; as well as with chemical synthesis of complex 3FTxs [43-45]. The utilisation of "mimotope" α-3FNTx fragments that bind nAChRs [46-48], including combinatorial high-affinity peptides (HAPs) [49] have provided valuable insight into toxin-receptor interactions, as well as allowing their use as epitopes to tag and study other membrane receptors such as AMPA and $GABA_B$ [50, 51].

The pioneering 3D structures of erabutoxin-b (EbTx-b) [52] and neurotoxin-α [53] from *Laticauda semifasciata* were a milestone in atomic-level analysis of α-3FNTxs. These were the first to describe the fine structural details at 2.5 - 2.75 Å of the three-finger protein fold (see Fig. 1 A; detailed in Section 5). These paralleled structural studies on the nAChR which began with electron microscopic analyses of *Torpedo* electric organ membranes that gradually progressed to higher resolution images (see review [54]), leading to the first-ever high-resolution image (at 2.7 Å) of an intact nAChR [55] (see Sections 9 and 14.2). The structure of the acetylcholine binding protein (AChBP), a soluble homolog of the neuronal α7 nAChR extra-cellular domain (ECD), isolated from snail glial cells, proved to be a landmark in structure-function studies of α-3FNTxs [56]. The past five years have seen impressive progress in high resolution structures of a variety of nAChR subtypes [55, 57-60] (see Section 9) and other membrane receptors including mAChRs [61] and $GABA_A$ receptors [62].

3 | Snake toxins modulating cholinergic neurotransmission

Snake venoms utilize a diverse array of structurally and functionally distinct toxins to target the cholinergic circuitry (see Fig. 1). Neuromuscular paralysis elicited by targeting the presynaptic and postsynaptic sides of the NMJ is the primary form of neurotoxicity resulting from envenomation

by the Elapidae family (including cobras, kraits, mambas, coral snakes, Australian elapids and sea snakes) as well as some true Viperidae and Colubridae species [21, 63, 64].

Postsynaptic neurotoxicity is characteristic of α-3FNTxs present as monomers (e.g. EbTx-b, Fig. 1A; α-BgTx, Fig. 1E) or dimers (e.g. irditoxin, Fig. 1G) and other unusual α-3FNTxs (e.g. candoxin, Fig. 1 H; WTX, Fig. 1 I) that competitively inhibit nAChRs at the NMJ, and these will be the focus of this review. Presynaptic activity at the NMJ is usually produced by the larger enzymatic toxin complexes (13 − 70 kDa) such as β-bungarotoxin (β-BgTx) (*Bungarus multicinctus*) and notexin (*Notechis scutatus*)(Fig. 1 D) [65, 66]. These toxins are associated with PLA_2 activity that hydrolyse phospholipids and disrupt membrane processes involved in the storage and release of acetylcholine (ACh) at motor nerve terminals [66-68]. Other secondary mechanisms of neurotoxicity have also been proposed for some PLA_2 toxins, including their action on muscle nAChRs [69-71] (detailed in Section 15).

Fasciculins (Fig. 1 C), a 3FNTx found in the venom of mambas (*Dendroaspis spp.*) act as potent inhibitors of acetylcholinesterase, which hydrolyses ACh resulting in the cessation of neurotransmission at cholinergic synapses [72, 73]. Mamba venoms also possess dendrotoxins, short peptides homologous to Kunitz-type serine protease inhibitors, that selectively inhibit voltage-gated potassium channels (Kv1.1, Kv1.2 and Kv1.6) at picomolar to low nanomolar concentrations and facilitate the release of ACh from the presynaptic terminus [74, 75].

Muscarinic acetylcholine receptors (mAChRs) which are G-protein coupled receptor complexes are also targeted with high affinity by a variety of muscarinic toxins, mostly from mambas (see reviews [76-78]). These are 3FNTxs (e.g. MTx-1; Fig. 1B) which exhibit distinct selectivity profiles for different mAChR subtypes and may act as competitive antagonists, allosteric modulators or agonists [78, 79]. Recently, α-3FNTxs WTX and α-cobratoxin (α-CbTx), both from

Naja kaouthia, were found to, respectively, modulate M1 and M3 mAChRs allosterically [80]; and inhibit M4 mAChRs [81].

Highly selective neurotoxins directed against nAChRs, derived from cone snails [82-85] and snakes [18, 19] contributed significantly to our understanding of neuronal nAChRs. κ-Neurotoxins, represented by κ-bungarotoxin (κ-BgTx) (*Bungarus multicinctus*), is a dimeric 3FNTx (Fig. 1 F) that is distinguished from other postsynaptic α-3FNTxs by their ability to inhibit neuronal α3 and α4 nAChRs, and inability to bind to muscle nAChRs [86]. The term 'κ-BgTx' was derived from 'kiliaris,' *Latin* meaning 'related to the eye,' since their activity was first demonstrated in the ciliary ganglion [87]. Furthermore, some monomeric and dimeric α-3FNTxs that interact with muscle nAChRs also extend their subtype selectivity to include neuronal nAChRs [19, 24] (see Table 2).

4 | Curaremimetic α-neurotoxins from snakes

α-Neurotoxins bind to orthosteric sites in postsynaptic nAChRs at the NMJ and competitively inhibit the binding of the endogenous neurotransmitter ACh, mimicking the action of d-tubocurarine and being widely referred to as "curaremimetic" toxins [19, 22, 24]. However, in comparison to d-tubocurarine, their binding affinity to muscle nAChRs (K_D ~10^{-9}–10^{-11} M) is 15- to 20-fold greater, and their interaction with muscle nAChRs is typically irreversible. Curaremimetic α-neurotoxins are present in Elapid, Viperid and Colubrid venoms [18, 21, 24].

Most snake α-neurotoxins adopt the conserved 3FTx scaffold, albeit with distinct structural and functional variability, which are discussed in this review. There are two noteworthy exceptions: waglerins from Wagler's pit viper (*Tropidolaemus wagleri*) contain just 22 − 24 residues and a single disulfide bridge [88], which also heralded the discovery of an α-neurotoxin from a Viperidae species, which produce typically hemotoxic venoms [89]. The other is azemiopsin from Fea's pit

viper (*Azemiops feae*) which consists of 21 residues and a C-terminal hexapeptide homologous to waglerins but is devoid of *any* disulfide bridges [90]. Interestingly, waglerins and azemiopsin have greater binding affinity for the α-ε over the α-γ and α-δ interfaces of muscle nAChRs [91] (see Section 11).

While α-BgTx may have been the first, α-neurotoxins are not exclusive to snakes. Conotoxins from marine cone snails which are short (12 − 30 residues) disulfide-rich compact molecules, show high affinity and selectivity for an extensive variety of ion channels and receptors, including numerous nAChR subtypes (see reviews [18, 83-85, 92, 93]). α-Conotoxins, with high selectivity for muscle nAChRs, may also distinguish between the different subunit interfaces of muscle nAChRs [84, 94].

5 | The versatile three-finger protein scaffold

The 3FPs are a superfamily of structurally related polypeptides distributed in diverse species across the eukaryotic kingdom, from sea urchins to humans [95, 96]. They are classified as the '*snake toxin-like protein superfamily*' in the Structural Classification of Proteins (SCOP) database [97], and as the '*uPAR/Ly6/CD59/snake toxin superfamily*' in the Protein Family (Pfam) database [98], with over 2500 sequences identified. 3FPs are characterized by a protein scaffold consisting of three β-stranded loops (akin to fingers) converging at a globular, hydrophobic core secured by four conserved disulfide bridges (see examples in Fig. 1). Their significant functional diversity is achieved by variations in their primary sequences, distinct pharmacophores and the length, twists, turns and plasticity of the loops. The location of additional disulfide bridges, as well as extensions of the N- and C-termini also contribute to their distinctive functions [17, 19, 22, 99].

The 3FP fold is adopted by endogenous eukaryotic proteins in a variety of animals that constitute either prey or predators for snakes [17, 100, 101]. The human genome encodes for ~45 proteins which contain one to three 3FP domains in their structure [102]: including the majority which are

tethered to cell membranes by a glycosylphosphatidylinositol anchor, with examples such as ly6 (lymphocyte antigen 6), lynx1 (Ly6/neurotoxin-1), uPAR (urokinase-type plasminogen activator receptor), Lypd6 (Ly6/PLAUR domain-containing protein 6) and PSCA (prostate stem cell antigen); and secreted forms such as SLURP1, SLURP2 (secreted ly6/uPAR-related protein 1 and 2) and PATE protein (prostate and testes expression protein)[17, 19, 100, 101]. Within this diverse superfamily, lynx1 (Fig. 1 J), lypd6, PSCA, SLURP1 (Fig. 1 K), SLURP2 and PATE protein, as well as a variety of snake 3FNTxs (shown in Fig. 1) act on and modulate nAChRs in the central and peripheral nervous systems, in addition to extra-neuronal sites including epithelial cells, lungs, immune system and reproductive tract [17, 19, 99-101].

Snake 3FTxs are found in venoms of *all* snake families, and are the predominant toxins in Elapid venoms, representing 75% - 95% of all toxins in the king cobra (*Ophiophagus hannah*)[103], mamba (*Dendroaspis angusticeps*)[104] and coral snake (*Micrurus tschudii*) [105] venoms. Given the breadth of the uPAR/Ly6/CD59/snake toxin superfamily, it is believed that snake 3FTXs have been recruited from genes encoding ancestral non-toxic proteins and selectively expressed in venom glands, with gene duplication and accelerated evolution driving the refinement of neurotoxic capability in 3FNTxs [6]. Further, 3FNTxs are likely involved in a coevolutionary "arms-race" with receptors and other molecular targets of their prey and predators, underpinned by positive selection, whereas other 3FTxs like cytotoxins that act via generalized non-specific mechanisms are constrained by negative selection [106, 107].

The snake 3FTx scaffold is composed of ~60 to 90 amino acid residues, and its three flexible loops form a flat "leaf-like" molecule with a slight concavity, the plane being determined by the extensive multi-stranded β-sheet structure (Fig. 4 F) [17, 22, 99]. The side-chains of canonical amino acids that constitute the functional site usually protrude from the concave side of the toxin (Fig. 4) [22,

108]. This structure is engineered by evolution to enable venomous animals to utilize a small number of structural templates to bind to a multitude of molecular targets [20, 108]. Consequently, snake 3FTxs have generated sweeping pharmacological actions in their prey and predators which include neurotoxicity, cytotoxicity, cardiotoxicity, coagulotoxicity, proteinase activity and acetylcholinesterase inhibition [17, 19]. Their scope of molecular targets include a variety of nAChRs, $GABA_A$ receptors [109-112], mAChRs [76, 78], α-adrenergic receptors [113, 114], β-adrenergic receptors [115], acid-sensing channels [116], platelet integrin $\alpha_{IIb}\beta3$ receptor [117], L-type calcium channels [118], potassium channels [119], sodium channels [120], and acetylcholinesterase [121, 122]. α-Neurotoxins that adopt the 3FTx structure are discussed further.

6 | Three-finger α-neurotoxins

α-3FNTxs are key components of neurotoxic snake venoms [22, 24]. They are broadly, and perhaps contentiously, classified into four *groups* based on their structural characteristics: short-chain α-3FNTxs (SC-α-3FNTxs), long-chain α-3FNTxs (LC-α-3FNTxs), non-conventional α-3FNTxs (NC-α-3FNTxs) and dimeric α-3FNTxs; as well as *atypical* 3FNTxs with variances in primary and tertiary structure (Table 1, Fig. 2) [19, 22]. Significant diversity in nAChR subtype selectivity, binding affinities and reversibility of action is evident even within the same α-3FNTx groups.

Largely, all α-3FNTxs inhibit the muscle nAChR with varying affinities, whilst LC-α-3FNTxs and NC-α-3FNTxs also inhibit some neuronal nAChR subtypes [17, 18, 20]. Dimeric α-3FNTxs appear to exhibit broader nAChR selectivity than that of their constituent monomeric α-3FNTx group, suggesting that dimerization is a strategy to achieve a wider target selection. For example, although SC-α-3FNTxs do not inhibit neuronal nAChRs, haditoxin [123] and fulditoxin [124], both dimeric α-3FNTxs composed of SC-α-3FNTx subunits, are active against both muscle and neuronal nAChRs (see Section 6.3). Interestingly, some α-3FNTxs such as α-BgTx, α-CbTx and WTX were

also shown to inhibit $GABA_A$ receptors which are pentameric ligand-gated channels of the same superfamily as nAChRs [109-111], as did waglerin-1 which is not a 3FNTx [125]. Thus, it is possible that other unknown molecular targets exist, especially for the less well characterised NC-α-3FNTxs which exhibit widely variable nAChR selectivity and affinity [126], with some such as WTX extending selectivity to target mAChRs [127], and others (e.g. bucandin (*Bungarus candidus*)) still without a clearly established molecular target [128]. Limitations in categorising α-3FNTxs within rigidly defined groups based solely on their structural characteristics is challenging, as evidenced by atypical 3FNTxs that are potent inhibitors of muscle nAChRs, such as pseudonajatoxin-a (*Pseudonaja textilis*), which has 117 amino acid residues and seven disulfide bonds [129]. Other atypical α-3FNTxs (see Fig. 2) are described below in Section 6.4.

6.1 | **Short-chain and long-chain three-finger α-neurotoxins**

Historically, based on the length of their polypeptide chains, α-3FNTxs have been categorized as either SC-α-3FNTxs (Type I) with 60-62 residues (6–7 KDa) and four conserved disulfide bridges; or LC-α-3FNTxs (Type II) with 66-75 residues (7–9 KDa), a longer carboxy-terminal tail, shorter Loop I, and an additional fifth disulfide bond between Cys30 and Cys34 at the tip of Loop II (see Table 1; Fig. 1 A, E; Fig. 2) [18, 22, 24, 34]. This classification is increasingly harder to adhere to with emerging α-3FNTxs with unique structural and functional properties, and different nomenclature and groupings have been proposed [7, 130, 131](see Section 7).

Both groups of α-3FNTxs bind with high affinity ($K_D 10^{-10}$–10^{-11} M) to muscle $(\alpha1)_2\beta1\gamma\delta$, as well its structurally homologous *Torpedo* nAChRs; whereas, LC-α-3FNTxs, but not SC-α-3FNTxs, are able to bind to neuronal α7 (K_D 10^{-9} –10^{-8} M) [132], α9 and α9/α10 nAChRs [133]; as well as to other pentameric ligand-gated $GABA_A$ receptors (K_D 5–20^{-6} M) [111]. High affinity binding of LC-α-3FNTxs to α7 nAChRs had been attributed to its fifth disulfide bridge in Loop II [134]; although

subsequently NC-α-3FNTxs with a fifth disulfide bridge in Loop I (e.g. candoxin)[126], dimeric SC-α-3FNTxs (e.g. haditoxin, fulditoxin) [123, 124], and novel α-3FNTxs without a fifth disulfide bond (e.g. Ω-neurotoxins)[135], have also been shown to inhibit α7 and other neuronal nAChRs.

6.2 | **Non-conventional three-finger α-neurotoxins**

NC-α-3FNTxs are α-3FNTxs with 62-68 residues and a fifth disulfide bridge in Loop I [126] (Fig 1 H, I). Some initially characterized members (e.g. WTX) had low toxicity (LD_{50} ~ 5–80 mg/kg), relative to typical α-3FNTxs (0.04–0.3 mg/kg), earning them the moniker "weak toxins" [136]. This was not universally the case, as γ-bungarotoxin (*Bungarus multicinctus*) is highly toxic [137], underscoring the functional diversity among this group [126]. For example, WTX [136] and Wntx-5 (*Naja sputatrix*) [45] have poorly reversible and weak (micromolar) interaction with muscle $(\alpha1)_2\beta1\gamma\delta$ and neuronal α7 nAChRs, while WTX also allosterically modulated mAChRs [127]. In contrast, candoxin exhibited reversible, nanomolar inhibition of muscle nAChRs [138, 139]. The biological activity and molecular targets of some NC-α-3FNTxs (e.g. bucandin) remain unknown [126, 128].

A phylogenetically distinct collection of rear-fanged snakes includes the Colubridae family, in which 3FTxs can constitute as much as 84 - 92% of venoms. Neurotoxicity from Colubrid venoms is attributed exclusively to NC-α-3FNTxs (see review [21]), which have distinctive characteristics including being longer (>75 residues; 8.5-10.7 kDa) with an elongated N-terminus (Fig. 2); and exhibiting taxon-specificity for bird or lizard nAChRs, which directly correlates with their diet [140, 141]. Furthermore, covalently linked heterodimeric NC-α-3FNTxs have also been identified in Colubrid venoms, including irditoxin (*Boiga irregularis*) (Fig. 1 G) and sulditoxin (*Spilotes sulphureus*).

6.3 | **Dimeric three-finger α-neurotoxins**

Snake neurotoxins existing as dimers was demonstrated over 35 years ago with κ-BgTx [142]. In the past decade, a plethora of dimeric α-3FNTxs have been discovered with novel structural and pharmacological characteristics that distinguish them from κ-BgTx (see Table 2). These include covalently-bound dimers with one [140] or two [143] disulfide bridges, and non-covalently bound dimers held together primarily by hydrogen bonding [123] or hydrophobic interactions [124].

Non-covalent homodimers, haditoxin and κ-BgTx share a similar quaternary structure with their monomeric units held in an antiparallel orientation through extensive hydrogen bonding between their Loop III β-strands [123, 144]. However, the monomeric subunits in the κ-BgTx dimer are LC-α-3FNTxs, and whereas they are SC-α-3FNTxs in dimeric haditoxin (see Table 2). The non-covalent homodimer, fulditoxin (*Micrurus fulvius*), is also composed of SC-α-3FNTxs, but held together primarily by 29 *hydrophobic interactions* between Loop II residues (unlike κ-BgTx and haditoxin)[124]. A homodimeric α-CbTx, with its monomeric subunits of LC-α-3FNTxs bound together by two intermolecular disulfide bridges [143, 145]; and irditoxin (*Boiga irregularis*) [140] and sulditoxin (*Spilotes sulphureus*) [146], heterodimers of two NC-α-3FNTx monomers linked by a single intermolecular disulfide bond, are examples of covalently bound dimers.

The impact of dimerization of snake 3FNTxs appears to be diversification of target selectivity by enabling novel structural conformations that allow interactions with new receptor subtypes which are not targeted by their monomeric constituents [19, 124]. κ-BgTx binds with high affinity to neuronal α3β2 as well as weakly to α7 and α4β2 nAChRs, but not to muscle nAChRs [86, 147]. Homodimers, haditoxin (inhibits muscle $(\alpha1)_2\beta1\gamma\delta$ and neuronal α7, α3β2 and α4β2 nAChRs) [148] and fulditoxin (inhibits muscle $(\alpha1)_2\beta1\varepsilon\delta$ and neuronal α7, α4β2 and α3β2 nAChRs) [124] (see Table 2), reveal a breadth of nAChR selectivity which is unusual for their monomeric subunit SC-α-3FNTxs that bind only to muscle nAChRs [149]. Similarly, dimerization not only retained

α-CbTx's ability to inhibit muscle $(\alpha1)_2\beta1\gamma\delta$ and α7 nAChRs, but extended its capacity to target α3β2 nAChRs [143, 145]. Detailed structure-function relationships that reveal the specific role of dimerization in generating expanded receptor selectivity are currently not known.

Covalently-linked heterodimers from Colubrids are examples of proteins that have undergone convergent evolution with taxa-specific neurotoxicity to effectively hunt birds and lizards, reflecting the diet of these arboreal species [21]. For example, irditoxin targeted avian muscle nAChRs with three orders of magnitude higher affinity than mammalian muscle [140]; while sulditoxin was lethal to lizards and non-toxic in mammals [146]. Dimeric α-3FNTxs are therefore a structurally and functionally heterogeneous group with expanded target selectivity.

6.4 | **Atypical three-finger α-neurotoxins**

In the past decade, many snake 3FNTxs have been described that have challenged conventional classifications founded on structure and underscored the risk of attempting to define function based on structural similarity. Some atypical 3FNTxs that have distinguishing structural variances, or primary sequence differences with implications for function, are discussed here.

Atypical LC-α-3FNTxs from the sea snakes | Two unusual 3FNTxs, Lc-a and Lc-b from the yellow-lipped banded sea krait *Laticauda colubrina* (Fig. 2) were classified as LC-α-3FNTxs based on sequence length (69 residues) and homology [150]. However, Lc-a and Lc-b lacked the fifth disulfide bridge at the tip of Loop II. Like most α-3FNTxs, both showed high affinity for *Torpedo* nAChRs (K_D ~ 0.02 nM) [150], but unlike LC-α-3FNTxs, they failed to inhibit α7 nAChRs; which was attributed to the absence of the Loop II disulfide bridge that was deemed crucial for binding α7 nAChRs [149]. LSIII from the banded black krait *Laticauda semifasciata*, a LC-α-3FNTx with five disulfide bridges, had an unusually short C-terminal tail consisting of just four residues after the terminal Cys (instead of nine as in α-BgTx and α-CbTx) (Fig. 2). LSIII was potent (K_D ~1.6

nM) at binding to *Torpedo* nAChRs, suggesting that the contribution to nAChR binding afforded by the C-terminal tail, is likely compensated by other functional residues [149].

Type-III neurotoxins from the brown snake *Pseudonaja textilis* | α-3FNTxs with four disulfide bridges, that are unique to the genus *Pseudonaja*, are amongst the shortest (57 − 58 residues) of what were structurally deemed to be SC-α-3FNTxs (Fig. 2) [151]. However, these α-3FNTxs lacked all the canonical residues of SC-α-3FNTxs for binding to nAChRs and shared only 40% identity with them (including the eight Cys residues), but produced neuromuscular paralysis and death in envenomed mice [151]. Phylogenetic analyses showed these toxins to be clustered distinctly from SC-α-3FNTxs and LC-α-3FNTxs, separate even from other Australian elapid α-3FNTxs, warranting a separate classification as Type III neurotoxins [7, 106](see Section 7).

Ω-neurotoxins from the king cobra *Ophiophagus hannah* | Ω-Neurotoxins, represented by Oh9-1, are structurally SC-α-3FNTxs, but lack the functional residues of typical α-3FNTxs critical for nAChR interaction [135]. Oh9-1 produced reversible postsynaptic neurotoxicity at low micromolar concentrations acting on muscle $(\alpha1)_2\beta1\varepsilon\delta$ and $(\alpha1)_2\beta1\gamma\delta$ nAChRs, as well as on α3β2 nAChRs, not usually a target for SC-α-3FNTxs. Oh9-1 selectively utilized ten residues spread over the three Loops, including two critical Loop II residues (Met25, Phe27) to interact with muscle and α3β2 nAChRs. Remarkably, the tip of Loop II in Oh9-1 was not involved in binding nAChRs unlike its indispensable role in most α-3FNTx interactions with nAChRs. Phylogenetic analysis revealed Ω-neurotoxins to be independent of α-3FNTxs, placing it in a new class [135].

Drysdalin from the white-lipped snake *Drysdalia coronoides* | Drysdalin and related α-3FNTxs from this Australian elapid are the longest (79 - 88 residues) of what could be classified as LC-α-3FNTxs, given the presence of an additional disulfide bridge in Loop II (Fig. 2) [130]. Nanomolar concentrations of drysdalin (comparable to typical LC-α-3FNTxs) were shown to inhibit muscle

$(\alpha1)_2\beta1\varepsilon\delta$ and α7 nAChRs irreversibly, and α9α10 nAChRs reversibly. This class, however, has long Arg- and Pro-rich C-terminal extensions (17 - 24 residues), the truncation of which led to total loss of activity at α9α10 nAChRs, and a 2.5-fold loss of potency at $(\alpha1)_2\beta1\varepsilon\delta$ and α7 nAChRs, with the inhibition also becoming reversible [130] (see Section 10).

Although drysdalin showed comparable neurotoxicity as typical LC-α-3FNTxs, it lacked three canonical residues essential for neuronal nAChR-binding (Phe29, Arg33, Arg36 in α-CBTx) [152, 153], and instead had Arg29, Leu33 and Ala36. Interestingly, 'restoration' of the canonical residues (i.e. Leu33Arg and Ala36Arg mutations) in drysdalin did not impact its potency, whereas the Arg29Phe mutation led to a complete loss of activity at α9α10 nAChRs and ~19-fold reduction in potency for α7 nAChR activity, underscoring the significance of a functional rather than sequence conservation of amino acid residues in the activity of drysdalin [130].

αδ-Neurotoxins from the Malayan krait *Bungarus candidus* | αδ-Neurotoxins (73 residues) were characterized from the same venom [154], from which candoxin that produced reversible neuromuscular blocking activity was previously described [138, 139]. αδ-Neurotoxins, which are structurally homologous to and equivalently toxic *in vivo* as α-BgTx, differed uniquely in their pharmacology in showing two orders of magnitude higher affinity for the α-δ interface over α-γ and α-ε interfaces of muscle nAChRs (see Section 11); as well being reversible in their activity on muscle $(\alpha1)_2\beta1\varepsilon\delta$, *Torpedo* $(\alpha1)_2\beta1\gamma\delta$, α7 and α3β2 nAChRs [154] (see Section 10). Molecular modelling and sequence comparisons with α-BgTx variants suggest that a probable reason for this "subsite-selectivity" maybe the lower content of positively charged residues in αδ-BgTxs (see Section 11) [154].

∑-neurotoxins from coral snake *Micrurus fulvius* | Dimeric fulditoxin (Fig. 2; Table 2) was shown to coalesce and form unique tetrameric complexes (consisting of four dimers) around two

Zn^{2+} atoms, with each Zn^{2+} atom coordinated with four His29 residues at the tip of Loop II [124]. This is the first report of a 3FP showing metal-binding capability. Interestingly, His29 is conserved in other 3FTxs identified by transcriptomic analysis of *Micrurus species* venom glands, suggesting that these 3FTXs may also undergo Zn^{2+}-coordinated tetramerization [124]. The functional implications of this tetramerization are unclear given that they occurred at concentrations higher than which were pharmacologically relevant.

Dimeric fulditoxin inhibited muscle $(\alpha1)_2\beta1\varepsilon\delta$ nAChRs at low nanomolar, and α7, α4β2 and α3β2 nAChRs at low micromolar concentrations. Like Ω-neurotoxins, the primary sequence of fulditoxin also lacks every canonical residue crucial for binding to muscle or neuronal nAChRs, indicating the presence of novel, yet undetermined, pharmacophores for nAChR recognition [124]. This led to the proposition that fulditoxin and other homologous *Micrurus* toxins belong to a new class of nAChR-targeting 3FNTxs named: Σ-neurotoxins [124].

Snakes utilize a common 3FP scaffold and vary amino acids to derive novel pharmacophores which bind to diverse molecular targets and produce a range of biological actions, underpinning functional evolutionary divergence of proteins [20, 99]. In contrast, structurally distinct protein scaffolds can also display functional similarity through *convergent* evolution [19], including waglerins, azemiopsin, phospholipase A_2, Ω-neurotoxins and Σ-neurotoxins, which have significantly diverse structures or novel sequences, but bind to the orthosteric binding sites of nAChRs [20, 99].

7 | Nomenclature of snake toxins

Against the backdrop of the rapid, technology-driven increase in the number of peptide toxins being discovered from animal venoms, King et al. (2008) highlighted the inconsistencies and confusion caused by the *ad hoc* naming of α-3FNTxs using trivial names, often based on the amalgamation

of the word “toxin” with a derivative of either the genus, species or both, of the snake from which the toxin was isolated [131]. A more systematic, rational and unified nomenclature was proposed that, for α-3FNTxs would use the prefix ‘α’ to denote an inhibitory action on nAChRs; followed by the snake’s family (e.g. *elapitoxin* if from the Elapidae); the genus and species are then indicated using single letters (e.g. Bm for *Bungarus multicinctus*); and lastly, numbers designate whether the isolated α-3FNTx is a short-chain (1), long-chain (2) or κ-neurotoxin (3), while any isoforms of the toxin would then be designated sequentially with alphabets (e.g. a, b, c) [24, 131]. In this schema, α-BgTx (from *Bungarus multicinctus* of the family *Elapidae*) would be referred to as *α-Elapitoxin-Bm2a.*

Based on phylogenetic analyses, Fry et al. (2003), designated SC-α-3FNTxs and LC-α-3FNTxs as Type I and II neurotoxins, and referred to a group of SC-α-3FNTxs from *Pseudonaja textilis* with novel sequences as Type III neurotoxins [7, 106]. Kini et al., (2018) have proposed that LC-α-3FNTxs be designated into three classes (1 to 3) according to the number of C-terminal tail residues after the last conserved Cys; and based on variations in canonical functional residues, be further grouped using alphabets (e.g. 1a and 1b; 2a - 2d; 3a - 3c) [130]. Accordingly, LC-α-3FNTx Classes 1, 2 and 3 were toxins with 4 - 5, 6 - 13 and 17 - 24 C-terminal residues, respectively. Although these proposed nomenclatures have not been universally adopted by researchers, with whom trivial names continue to be popular, the inclusion of the systematic classification of any new α-3FNTx (in addition to its trivial name) must be encouraged.

8 | Assays and screens for α-neurotoxin activity

Early assays for neurotoxicity involved the injection of α-neurotoxins into animals followed by observation for clinical signs of flaccid paralysis and post-mortem examination if death ensued. Being competitive antagonists of postsynaptic muscle nAChRs at the NMJ, α-3FNTxs are

effectively characterized using isolated skeletal muscle–nerve preparations from chick (chick biventer cervicis muscle [155]) and rodent (rat or mouse phrenic nerve-hemidiaphragm [156]) in organ bath assays [24, 157-159]. This can also be a useful screen for bioassay-driven chromatographical purification of α-neurotoxins [124, 159, 160].

The presynaptic, postsynaptic or myotoxic effects of α-neurotoxins can be clearly distinguished by the chick biventer muscle with its focally and multiply innervated muscle fibres which, respectively, mediate twitch responses evoked presynaptically by electrical nerve stimulation and the contractile responses produced by exogenous nAChR agonists such as ACh and carbachol by activation of postsynaptic nAChRs [22, 24]. Postsynaptic α-neurotoxins would inhibit both, the responses to nAChR agonists as well as nerve stimulation; whereas presynaptic neurotoxins would abolish only nerve-evoked twitches without affecting agonist-induced responses. Contractions evoked by direct depolarization of skeletal muscle by potassium chloride would only be affected by myotoxic activity that disrupts the muscle's structural integrity [157]. Likewise, *in vivo* characterization of α-neurotoxins in anaesthetized rodents is also possible [138].

Definitive binding of α-neurotoxins to nAChRs may be quantified by the competitive binding of radio-labelled toxins (e.g. ^{125}I-labeled α-BgTx) to nAChR-rich membranes from *Torpedo* electric organs; and experimental conditions judiciously varied to include the presence and absence of various agonists and antagonists to delineate pharmacological properties such as competitive and non-competitive binding and reversibility of binding [161]. However, binding assays do not necessarily provide evidence of functional blockade of nAChRs [24]. Electrophysiological approaches where the ability of α-neurotoxins to inhibit currents or ion fluxes induced by agonists in heterologously expressed nAChRs in oocytes are invaluable for detailing function, including selectivity for nAChR subtypes [160, 161]. While traditional two-electrode voltage clamping

approaches are time- and labour-intensive, newer automated, high-throughput screening systems are routinely being employed for this purpose [162, 163]. For a comprehensive review of screening of venoms and target identification of toxins see Vetter et al. (2015) [160].

9 | Nicotinic acetylcholine receptors

Aided by the discovery of α-BgTx, and an abundant resource from the electric ray (*Torpedo* spp.) and eel (*Electrophorus* spp.), the nAChR is the most comprehensively studied receptor to date [31]. The discovery of the AChBP with 27% identity to the ECD of human α7 nAChRs led to innovative AChBP–α7 nAChR ECD chimeras (with >60% identity), and studies of ligand binding to nAChRs [56]. Partial structures of other nAChRs including the ECD of single subunits of rodent α1 [164], and human α9 [165, 166] nAChRs; as well as human α2 nAChR ECDs as a homopentamer (which is not a known physiological assembly) [60] were then detailed. These were followed by emerging 3D structures of intact human α4β2 [58, 167] and α3β4 nAChRs [59], as well as the *Torpedo* $(\alpha1)_2\beta1\gamma\delta$ in complex with α-BgTx [55]. The latter, at 2.7 Å resolution (Fig. 3 A, B), was a significant improvement from the previous 4.0 Å structure [54]. Collectively these studies have provided the most detailed examinations of the nAChR structure, gating mechanism, ligand-binding and direct validation of historical mutational studies.

The nAChR is a hetero- or homo-pentameric transmembrane allosteric protein (MW ~290 KDa) that evokes fast ionic responses to endogenous ACh (see reviews [54, 57, 168-171]). It is a member of the "Cys-loop" pentameric ligand-gated ion channel superfamily, that also includes $GABA_A$, $5HT_3$ and glycine receptors and zinc-activated ion channels (reviewed in [172, 173]). Five homologous subunits associate pseudo-symmetrically around a central transmembrane ion channel in the nAChR (Fig. 3 A-C). Each subunit has a large amino-terminal ECD that contributes to the formation of orthosteric ligand-binding binding sites at subunit interfaces between a primary (α)

and a complimentary (α or non-α) subunit (Fig. 3 B, D). A transmembrane domain comprising of four α-helices, of which the second (M2) (from each subunit) lines the ion channel pore (Fig. 3 C), and an intracellular domain of variable structure (Fig. 3 A) complete the nAChR assembly [54, 57].

The combinatorial assembly of 17 nAChR subunits (α1 to α10, β1 to β4, δ, γ or ε) generates a diversity of nAChR subtypes with distinct pharmacological, physiological and clinical significance [168-170]. The postsynaptic skeletal muscle nAChR is of the stoichiometry $(\alpha1)_2\beta1\varepsilon\delta$ in its adult form, and $(\alpha1)_2\beta1\gamma\delta$ in fetal form as well as in the homologous *Torpedo* nAChR [174]. Endogenous ACh, released from nerve terminals following depolarization, binds simultaneously to orthosteric binding sites at the α1-δ and α1-ε or α1-γ subunit interfaces (Fig. 3 B, E) to activate the nAChR; triggering sequential conformational changes resulting in the opening of the cation channel and leading to depolarisation of the motor end plate and eventually, contraction of the muscle [169]. Neuronal nAChRs comprise of various pentameric combinations of α2 to α10 and β2 to β4 subunits, and play significant physiological roles in the central nervous system including cognition, memory, pain perception and addiction, as well as in extra-neurological locations where they modulate key cellular signalling pathways (see reviews [168, 175]). Neuronal nAChRs underlie the pathophysiology of a several neurological and neuropsychiatric conditions, making them important therapeutic targets [176, 177].

The orthosteric ligand-binding subunit interfaces are formed by six polypeptide loops: Loops A, B and C from the primary α1 subunit, and Loops D, E and F from the complementary δ, γ or ε subunit. Thus, there are three distinct interfaces (α1-δ and α1-ε or α1-γ) in muscle nAChRs. At the core of the binding interface is the 'aromatic box' with highly conserved aromatic amino acids including Tyr93 (Loop A), Trp149 (Loop B), Tyr190, and Tyr198 (Loop C) from the primary, and Trp57 (Loop D) from the complementary, interfaces (numbering relates to the α1-γ interface; see Fig. 5

F). These aromatic residues stabilise the ligand through critical cation-π and hydrophobic interactions, while other residues from Loops A to F also contribute to varying degrees with ligand-specific interactions [54, 57, 169].

10 | Reversibility of snake α-neurotoxin actions

The neuromuscular blockade produced by most α-neurotoxins, especially LC-α-3FNTxs, is poorly reversible [178]. SC-α-3FNTxs have shown some variability [24], with CM10 and CM12 (*Naja haje annulifera*), S5C10 (*Dendroaspis jamesoni*) and LSIII (*Laticauda semifasciata*) eliciting completely reversible neuromuscular blockade *in vitro* [179]. The mechanisms underlying reversible interaction of α-3FNTxs with muscle nAChRs is complex and not well understood. Given that the off-rates of LC-α-3FNTxs from nAChRs are very slow [180], with a dissociation half-time for the [^{3}H]α-3FNTx–*Torpedo* nAChR complex to be ~60 h [181]; the scope of organ bath or electrophysiological experimental designs (lasting minutes to a few hours) to study reversibility α-3FNTxs is limited [124].

SC-α-3FNTxs were found to associate six to seven-fold faster, and dissociate five to nine-fold faster, compared to LC-α-3FNTxs, in binding studies with *Torpedo* nAChRs [182]. Past studies have proposed that the α1-subunit Loop C region is inefficient in binding to SC-α-3FNTxs unlike it does to LC-α-3FNTxs [183]; and that the higher proportion of hydrophobic amino acids in SC-α-3FNTxs may underpin their reversible interaction with nAChRs [184]. Interestingly, Silva et al. (2019) and showed that SC-α-3FNTxs, but not LC-α-3FNTxs, dissociated readily from human muscle nAChRs *in vitro*; whereas both bound irreversibly to rodent muscle [185]. Consequently, they infer that SC-α-3FNTxs have a minimal role in neurotoxicity following snake envenomation.

Nonetheless, it must be noted that the reversibility or irreversibility of α-3FNTx-induced neuromuscular blockade cannot simply be attributed to their binding affinities to nAChRs. This is

evident from electrophysiological studies which showed that α-BgTx (IC_{50} ~ 5 nM) produced irreversible blockade of muscle $(\alpha1)_2\beta1\gamma\delta$ nAChRs, whereas the NC-α-3FNTx, candoxin (IC_{50} ~10 nM) [139] and dimeric SC-α-3FNTx, fulditoxin (IC_{50} ~27 nM) [124] produced reversible blockade. Furthermore, another NC-α-3FNTx, WTX, which is a 1000-fold weaker antagonist of $(\alpha1)_2\beta1\gamma\delta$ nAChRs, is almost irreversible in its action [136].

It has been proposed that the reversibility of α-3FNTx action on muscle nAChRs may be associated with a specific area of interaction on the α-3FNTx, distinct from its pharmacophore [179]. For instance, the absence of the polar / hydrophilic Asp31 in many reversible α-3FNTxs including candoxin and fulditoxin [124, 138] has been attributed to their easy reversibility, which also supports the observations that reversible α-3FNTxs have a higher prevalence of hydrophobic residues [184]. Contradicting this, recently characterized αδ-neurotoxins which produce reversible blockade of muscle nAChRs have the equivalent Asp in their primary sequence [154]. Interestingly, it was shown that drysdalin, an atypical toxin with a long C-terminal extension, bound irreversibly to muscle nAChRs. However, drysdalin became reversible in its action when its C-terminal tail was truncated, revealing that reversibility may be associated with C-terminal tail residues [130].

11 | Binding site-selectivity of α-neurotoxins in muscle nicotinic acetylcholine receptors

The muscle nAChR has distinct ligand-binding interfaces between α1-δ, α1-ε and α1-γ subunits [186] which determine variable affinities and selectivity for agonists and antagonists [187, 188]. Typically SC-α-3FNTxs and LC-α-3FNTxs cannot effectively distinguish between these distinct ligand-binding interfaces and bind with equal affinity [189]. Animal toxins which do, do exist. For example, in the muscle nAChR, conotoxin MI from *Conus magus* showed 10,000-fold selectivity

for the α1-δ site [190]; and Viperid toxins, waglerin and azemiopsin showed, respectively, a 2000- and ~30-fold greater affinity for the α1-ε site [90, 191].

A SC-α-3FNTx, NmmI (*Naja mossambica*) displays an order of magnitude higher affinity for the α1-γ or α1-δ interfaces (K_D ~140 pM) than for the α1-ε site (K_D ~130 nM); and this selectivity has been shown to be dependent on nAChR γ-subunit residues Pro175 and Glu176 [192]. Preliminary studies on the NC-α-3FNTx candoxin also revealed that it inhibited the muscle $(\alpha 1)_2\beta 1\gamma\delta$ nAChR with a biphasic concentration-response curve corresponding to a high affinity (2.2 nM) and low affinity (98 nM) binding sites, suggesting that it may have differential affinities for the α1-γ or α1-δ interfaces [138]. Furthermore, mutations Lys23Glu and Lys49Glu in α-CbTx caused a differential lowering of binding affinities at α1-γ or α1-δ sites [134], as did the Lys27Glu mutation in NmmI which affected binding at the α1-γ site more than at the α1-δ site [193]. Interestingly, position 29 in candoxin (homologous to positions 23 in α-CbTx and 27 in NmmI) is already occupied by Glu instead of Lys as found in α-CbTx and NmmI, suggesting a possible role for Glu29 in conferring differential interface selectivity by candoxin [138].

More recently, Elapid αδ-neurotoxins which are structurally related to α-BgTx, showed two orders of magnitude higher affinity for the nAChR α-δ, over α-γ and α-ε interfaces [154]. This preferential selectivity for the α-δ interface has been attributed to three key differences between them and α-BgTx: (i) substitution of α-BgTx's canonical Loop II Phe32 by the more bulky Trp residue (see Fig. 2) that likely changes the confirmation of αδ-neurotoxin's Loop II region; (ii) deletion in αδ-neurotoxin of the functionally important Ala7 found in α-BgTx's Loop I, which likely interferes with Loop I residues in interacting with nAChRs (see Section 14.2); and, (iii) replacement of the

canonical Arg25 in α-BgTx's Loop II by Thr in αδ-neurotoxin, thus removing an important positive charge for toxin binding [154].

12 | Structural stability of the three-finger protein fold

The 3FP fold is a remarkably stable molecule that offers versatility for the uPAR/Ly6/CD59/snake toxin superfamily to adopt diverse biological activities [17, 19, 20]. Several conserved amino acid residues contribute to the correct folding of the polypeptide chain and structural integrity of the 3FP backbone, contributing indirectly to interactions with molecular targets [17, 22, 99]. The eight Cys residues that form the four conserved disulfide bridges in the core region sustain the overall architecture of the 3FP (Fig. 2), and their absence renders the protein dysfunctional [194].

An aromatic residue, Tyr25 (EbTx-a numbering), or a homologous residue Phe27, is conserved in most 3FPs and is necessary for proper protein folding and stability of the anti-parallel β-sheet structure [99, 128]. Other residues, Gly42, Pro44 and Pro48 were also important to maintain the 3FP conformation, whereas Gly40 enabled close packing of the protein [22, 123, 140]. Likewise, the conserved Ser8 in EbTx-a is believed to be crucial in stabilising the orientation of side chains in Loop II, thus indirectly contributing to its function [41]. Some charged residues, Arg39 in EbTx-a and Asp60 in α-CbTx for instance, were shown to stabilize the native conformation of the 3FP by forming salt links with the C- or N- terminus of the toxin [123, 195].

13 | Functional considerations of the overall three-finger α-neurotoxin protein fold

The disulfide bridge-rich core region of 3FPs is the fulcrum from which the three loops extend and is indispensable for structural integrity. However, this region may also interact with the lipid bilayer adjacent to nAChRs; and undergo 'membrane catalysis,' where hydrophilic and positively-charged

side chains of the toxin's core region formed hydrogen bonds with the polar head groups of the lipid bilayer [196, 197], favourably positioning Loop II for binding to nAChRs [24, 196].

Key differences in structure between SC-α-3FNTxs and LC-α-3FNTxs include an extra disulfide bridge at the tip of Loop II, a longer C-terminal tail and a shorter Loop I in LC-α-3FNTxs (compare Fig. 1 A and E); which underpin significant functional variations [17, 20]. The presence of a helix-like segment at the tip of Loop II established by the fifth disulfide bridge in LC-α-3FNTxs (Fig. 1 E), has been deemed vital for high affinity binding to α7 nAChRs [149, 198]. It has also been found to be a dynamic entity which permits LC-α-3FNTxs to adopt alternate conformations enabling their binding to muscle and α7 nAChRs [199]. Accordingly, α-3FNTxs which lack the Loop II disulfide bridge, including SC-α-3FNTxs, atypical LC-α-3FNTxs and most NC-α-3FNTxs (which have the fifth disulfide bridge in Loop I), have weak affinity (K_D ~3–22 μM) for α7 nAChRs [126, 149].

Interestingly, a NC-α-3FNTx, candoxin, was the first example that lacks this critical Loop II disulfide bridge, but blocks α7 nAChRs in nanomolar concentrations, suggesting that it utilizes an alternate pharmacophore for this interaction [139]. In exploring the role of the Loop I disulfide bridge in NC-α-3FNTxs and mammalian Ly6 proteins, it was found that its disruption resulted in loss of function for the Lynx1 protein [200], but not for WTX interaction with nAChRs [80]. Other α-3FNTxs which lack the Loop II disulfide bridge but interact with α7 and other neuronal nAChRs include Ω-neurotoxin [135], and dimeric SC-α-3FNTxs haditoxin [123] and fulditoxin [124]. With dimeric α-3FNTxs, it is believed that dimerization diversifies target selection by enabling new structural conformations to permit interactions with various receptor subtypes [19, 124].

Mutational studies by Menez and co-workers identified a significant role for Loop I of SC-α-3FNTxs, but not LC-α-3FNTxs, in binding to the muscle nAChR (Fig. 4 A-C) [42, 201], which made sense given the longer Loop I of SC-α-3FNTxs. However, recent structural studies have

clearly recognized critical interactions of Loop I residues in LC-α-3FNTxs with nAChR proteins (Fig. 4 D, E; Fig. 5 E, F) [55, 164, 202], explained by the flexibility of the toxin's three loops and Loop C of the nAChR binding interface, which enabled conformational adaptations to allow binding [17, 55]. Further, the longer C-terminal tail of LC-α-3FNTxs has also been shown to stabilize binding [17, 18], with a more definitive role of the C-terminal tail in determining selectivity for nAChRs (e.g. drysdalin and selectivity for α9α10 nAChRs) also described [130]. These are detailed in Section 14.

14 | Molecular mechanisms of three-finger α-neurotoxin function

Attempts to understand structure-function relationships between α-3FNTxs and nAChRs began as early as the 1970s, with chemical modification studies and cross-linking of α-3FNTxs with nAChRs using bifunctional or photoactivatable reagents (see review [18]). These were followed by studies on heterologously expressed and chemically synthesized α-3FNTxs [42, 44, 45, 203]; studies of short mimotope peptides of α-3FNTx; combinatorial phage-display HAPs [49, 204, 205]; comparison of nAChRs from α-3FNTx-resistant species and nAChR subtypes which do not bind α-3FNTxs [206, 207]; engineering of α-3FNTxs by swapping or integrating pharmacophores [43, 208]; and, high resolution 3D structures of α-3FNTxs in complex with nAChRs [57]. In the light of conclusive atomic-level data on the binding of α-3FNTxs with nAChR models from high-resolution structural studies, this review will focus on reconciling these findings with historical mutagenesis studies and insights from combinatorial α-3FNTx-binding HAPs, as well as account for the differences between the various structures of α-3FNTx-nAChR complexes.

14.1 | Reconciling mutational studies with emerging structures of toxin-receptor complexes

Mutagenesis studies have established that α-3FNTxs utilize a *common binding core* of canonical amino acids to interact with key conserved residues at the nAChR ligand-binding interface; while

other residues specific for either SC-α-3FNTxs or LC-α-3FNTxs stabilize the interaction and/or confer nAChR subtype selectivity [132, 161]. The common binding core of α-3FNTxs for binding to muscle $(\alpha1)_2\beta1\gamma\delta$ nAChRs are predominantly positively charged and aromatic residues: Lys27, Trp29, Asp31, Phe32, Arg33 and Lys47 (EbTx-a numbering) (Fig. 4 A-C) [132, 193].

In particular, the highly conserved Arg at the tip of α-3FNTxs' Loop II (Arg33 in EbTx-a and α-CbTx; Arg36 in α-BgTx) (Fig. 4 A-E) is critical for toxin binding to nAChRs due to its positively charged guanidinium group which mimics the action of ACh [209]. The vital role of Arg33/36, as well as a more significant role for Phe32 than previously described, were confirmed by structural studies of α-CbTx in complex with AChBP [210]; as well as α-BgTx in complex with a chimera of AChBP and α7 nAChR [202], ECD of nAChR α1-subunit [164], ECD of the nAChR α9-subunit [165] and the intact *Torpedo* $(\alpha1)_2\beta1\gamma\delta$ nAChR [55] (discussed below).

Since conserved residues were believed to have comparable function among α-3FNTxs [45], a plausible explanation for the variable nAChR affinities of the poorly characterized NC-α-3FNTxs, was simply based on the presence or absence of canonical residues identified by mutagenesis in EbTx-a and α-CbTx, [126]. Accordingly, the weak inhibition of muscle nAChRs by cobra NC-α-3FNTxs (WTX [136]; Wntx-5 [45]) was attributed to the presence of just three canonical functional residues (Lys27, Lys47, Arg37). However, highlighting the limitations of this approach, recent mutation studies of WTX revealed that a unique pair of positively charged Arg31 and Arg32 in Loop II, not homologous to the canonical Arg33 in EbTx-a and α-CbTx, were critical for WTX's interaction with nAChRs and mAChRs [80].

Mutagenesis studies also established that the canonical functional residues in α-3FNTxs for binding nAChRs were located on the *concave surface* of the molecule (Fig. 4 F) [211], which was supported by the α-CbTx-AChBP structure that showed Loop C of the AChBP ligand-binding site entirely

wrapped around by the concave face of α-CbTx [210]. Recent structures of α-BgTx-nAChR complexes have found additional residues on the *convex surface* of Loop II (Lys38, Val39, Val40) and C-terminal tail (His68, Pro69, Lys70) to contribute to binding (Fig. 4 E; Fig. 5 E, F) [55, 164, 202].

Supplementing the role of the common binding core were *residues specific for SC-α-3FNTxs or LC-α-3FNTxs* for nAChR recognition (Fig. 4 A-C); which are, in EbTx-a (SC-α-3FNTx): His6, Gln7, Ser8, Ser9 and Gln10 of Loop I and Tyr25, Gly34, Ile36 and Glu38 of Loop II [42, 201, 211]; and in α-CbTx (LC-α-3FNTx): Arg36 of Loop II and Phe65 of the carboxy-terminal tail [212]. In contrast to Loop I of EbTx-a (consisting of 13 residues), Loop I of α-CbTx (consisting of 10 residues) was deemed too short to be accessible to the receptor and hence its lack of involvement in toxin binding [212, 213]. This was contradicted by the structure of the α-CbTx-AChBP complex that found Thr6, Pro7 and Ile9 in α-CbTx's Loop I to contribute to binding [210]. Furthermore, at least three complexes have shown the involvement of α-BgTx's Loop I (consisting of 12 residues), including Thr6, Ala7, Thr8, Ser9, Pro10 and Ile11 in binding to the nAChR α1-subunit (Fig. 5 E) [164], pentameric ECD of an α7-nAChR-AChBP chimera [202], and the intact *Torpedo* nAChR [55]; as well as with short cognate peptides of the *Torpedo* nAChR Loop C region [214].

The involvement of the long C-terminal tail of LC-α-3FNTxs in stabilizing their interaction with nAChRs has been confirmed in α-CbTx by mutagenesis (Phe65) [134], and in the α-CbTx-AChBP complex (Phe65, Arg68) [210]. It has also been established in complexes between α-BgTx and nAChR cognate peptides (Lys70 and Arg72) [214], and α-BgTx and nAChRs (His68, Pro69, Lys70) [55, 164, 202]. The role of the C-terminus of LC-α-3FNTxs varies widely, with minimal as in the case of LSIII with a short C-terminal tail (see Fig. 2) [149]; to substantial, as with the long C-terminal tail of drysdalin determining selectivity for α9α10 nAChRs [130].

14.2 | **Reconciling the differences between structures of toxin-receptor complexes**

Structural studies of synthetic peptides containing the toxin-binding sequences of nAChRs have long been utilized to explore how α-3FNTxs bound to nAChRs [199, 204, 214]. The identification, from a combinatorial phage-display library, of an α-BgTx-binding, 13-residue HAP homologous to Loop C of α1 and α7 nAChRs [215], led to the design of a peptide library to achieve even higher affinities (IC_{50} ~ 2 nM) by amino acid substitutions [49, 204, 216, 217]. Complexes between α-BgTx and HAPs revealed the tips of Loops I and II and the C-terminal tail showing conformational rearrangements to enable a snug fit of the toxin to the HAP [49, 204, 205]. Broadly, these findings have been corroborated by recent structures of α-BgTx in complex with the pentameric ECD of an AChBP-α7 nAChR chimera [202] and the ECD of single nAChR subunits α1 [164] and α9 [165]. Interestingly, a previously unknown, albeit limited involvement of α-BgTx's Loop III in receptor binding has been shown by the structure of α-BgTx bound to the intact *Torpedo* nAChR [55]. Limitations in making direct comparisons about the points of contact of α-BgTx binding to a 13-mer peptide, and more complex nAChR models must be taken into account [199, 214].

The soluble AChBP enabled insight of its binding with α-CbTx, via molecular modelling [198], and a 4.2 Å crystal structure [210]. It also enabled the design of a 'humanised' chimera of AChBP and the α7 nAChR ECD (with 64% sequence identity) (3.5 Å) to study α-BgTx binding [202]. These revealed five toxin molecules associated with the pentameric complex, with each toxin bound perpendicularly to the AChBP axis, and the tips of Loop II plugging into the ligand-binding interfaces (Fig. 5 A, B); with the C-terminal tail and Loop I making additional interactions at the receptor surface. From the receptor side, multi-point attachments were made with α-BgTx, mainly by Loop C, assisted by Loops A and B of the principal subunit, as well as Loops D and F from the complementary subunit [202, 210]. Being pentamers, the structures of the α-BgTx-AChBP-α7

chimera and α-CbTx-AChBP complex enabled toxin binding to an interface comprising of the principal and complimentary subunits unlike complexes with HAPs. However, AChBP represents only the ECD of nAChRs, and lack any conformational changes afforded by linking to the transmembrane and intracellular domains of an intact nAChR [55].

Structures of complexes between α-BgTx and single ECDs of mouse α1 subunit (1.94 Å) [164] or human α9 subunit (2.7 Å) [165] were limited by the absence of the complimentary interface in interactions with α-BgTx; which moved the toxin ~4.5 Å closer towards the binding site. The α-BgTx-α1 subunit complex was further limited by a mutation (Trp149Arg) required to achieve crystallization, even though Trp149 is known to be important for toxin binding. Despite this, the previously described consensus on toxin-binding prevailed, with Loop C of the α1 subunit wrapped around and interacting with α-BgTx's Loops I, II and C-terminus. The α-BgTx-α9 subunit complex too found similar interactions between the toxin's key residues and homologous nAChR aromatic residues as seen in the α-BgTx-α1 subunit complex, as well as with the α-BgTx-AChBP-α7 chimera structure [165]. Distinctively, in the α-BgTx-α1 subunit complex, extensive interaction of the toxin with an Asn-linked oligosaccharide in the Cys-loop region (found only in *Torpedo* and muscle nAChR subunits) was observed [164], which was not seen in the α-BgTx complex with the α9 subunit and AChBP-α7 chimera. This resulted in a slight (12 - 22°) clockwise rotation in orientation of the toxin at the binding interface in the structures lacking the glycosylation site [165].

Collectively, the complexes of α-BgTx with the α1 subunit, α9 subunit and AChBP-α7 chimera found the canonical Arg36 and Phe32 of α-BgTx's Loop II form an elaborate cation-π sandwich with Tyr198, Tyr190 and Tyr93 (or equivalent residues) at the core of the receptor ligand-binding pocket (see Fig. 5 D, E) [164, 165, 202]. The guanidium group of Arg36 at the tip of α-BgTx's Loop II occupies the same location as the nitrogen of ACh and other agonists, effectively acting as

a competitive antagonist [202]. Importantly, a '*tetrad of interaction*" vital for high affinity binding (Fig. 5 D), consisting of α-BgTx's Loop II Asp30, Arg36 and Phe32, together with the α1 subunit Loop C's Tyr190 (Tyr184 in the AChBP-α7 chimera) were identified in all three structures [164, 165, 202]. In contrast, the α-CbTx-AChBP complex diverged markedly, with Arg33 (Arg36 in α-BgTx) lodged against the complimentary subunit; and Phe29 (Phe32 in α-BgTx) instead occupying the center of the ligand-binding pocket with an alternate positively charged residue (Arg36) involved in cation-π interactions with AChBP's Loop C Tyr185 (Tyr190 in α1 nAChR) [210].

Arguably, the last word yet, comes from the most recent, highest resolution to date (at 2.7 Å) structure by Hibbs et al. (2020) [55], which provides atomic scale information on α-BgTx binding to an intact *Torpedo* $(\alpha1)_2\beta1\gamma\delta$ nAChR receptor and stabilizing its closed state conformation. About 25% of the total surface of α-BgTx (~1100 $Å^2$) is buried in the *Torpedo* receptor, which is less than that reported for complexes with the pentameric human α7-AChBP ECD chimera (~2000 $Å^2$) [202], and single mouse α1 subunit ECD (~1780 $Å^2$) [164]. This is explained in the α1 subunit ECD by absence of the complimentary subunit enabling closer encroachment by the toxin's Loop II into the binding pocket [165]. The Hibbs study confirmed the vital role of a large N-linked glycan arising from Asn141 in the Cys-Loop region (Cys128-Cys142) which, together with Loop C, formed the key interaction points with the toxin's Loop I and Loop II, respectively. The absence of the Cys-Loop glycan in the AChBP and α9 subunit takes away an important determinant of toxin binding which likely influences the variations observed between these structures.

The Hibbs structure also shows Loop F from the complimentary subunit, not reported by previous structures, scaffold α-BgTx's base and top of Loop II while making limited interactions with Loop III. While the concept of the canonical residues forming a 'tetrad of interaction" [164, 202] is not disputed by Hibbs et al. (2020), a broader coalition of aromatic residues including Tyr93, Tyr190,

Tyr198 and Trp149 from the primary α1 subunit, and Trp57 from the complimentary δ subunit (Trp55 in γ subunit) is shown in critical interactions with α-BgTx's Arg36 and Phe32. Importantly, a "*cation-π sandwich*" comprising Tyr198–Arg36–Phe32 (of the α1 subunit and α-BgTx) has been deemed central to toxin binding to *Torpedo,* muscle and α7 nAChRs [55]. Lastly, in contrast to agonists which stabilize a compact, tightly packed down Loop C which translates into the opening of the ion channel, the larger α-BgTx acts as an antagonist by prising open Loop C and preventing the compaction of the ligand-binding pocket [55].

14.3 | **Nicotinic acetylcholine receptors resistant to α-neurotoxins**

Structure-function relationships of α-3FNTxs have also been explored by analysis of muscle nAChRs of animal species inherently resistant to snake α-neurotoxins (e.g. mongoose, cobra) [218-220]. These studies found the substitution, in α-3FNTx-resistant nAChRs, of key aromatic and Pro residues of Loop C important for toxin binding [219]; and N-linked glycosylation of Asn residues in Loop C which imparts immunity to α-3FNTxs [207, 220]. Working on this concept, an α-3FNTx-resistant nAChR α3 subunit could be converted to an α-3FNTx-sensitive subunit (IC_{50} ~ 20 - 40 nM) by the exchange of just five residues in its Loop C, with the corresponding residues from α7 nAChRs [221]. This strategy has also succeeded with non-α subunits of nAChRs [205].

To further delineate the minimum pharmacophore for α-BgTx-sensitivity, another study found the double mutation of Lys189 and Ile196 in the α-3FNTx-refractory α4 subunit, with Phe189 and Pro196 as found in α-3FNTx-sensitive human α1 subunit, conferred them with nanomolar (K_D ~100 nM) affinity for α-BgTx binding. A third mutation Lys153Gly further improved affinity (K_D ~22 nM) [206]. This is corroborated by the Hibbs structure of the *Torpedo* nAChR, where Pro197 (Ile196 in α4) packs against Tyr198 of the *cation-π sandwich* to stabilize toxin-binding; whereas the aromatic Tyr189 (Lys189 in α4) directly interacts with the toxin's Loops I and II; and a Lys in

this position would clash sterically with toxin binding (see Fig. 5 F) [55]. A significant application of this strategy was the introduction of α-BgTx-binding pharmacophores into membrane proteins not usually the target of α-3FNTxs to tag, localize, isolate and characterize new proteins [205], as has been done with genetically-engineered AMPA and $GABA_B$ receptors [50, 51, 161].

15 | Role of α-neurotoxins in snakebite envenomation and implications for treatment

The global burden of snakebites, classified as a Category A Neglected Tropical Disease by the World Health Organization in 2017, is estimated to cause 1.2 to 1.8 million envenomings, 400,000 amputations, over 1.2 million years of life lived with disability and about 100,000 deaths every year. There are also poorly quantified long-term physical and psychological effects and substantial economic impact on vulnerable populations in the developing world [222-227].

The predominant clinical symptoms are often specific for the species of snake involved, with bites of Elapids being typically associated with neurotoxicity. Neurotoxins are also present in some Viperid and Colubrid venoms, with unusual neurotoxicity reported in human envenomation by the Sri Lankan Russel's viper (attributed to presynaptic PLA_2 toxins) [228], and unique prey-specific neurotoxicity produced by Colubrid α-3FNTxs [21]. The classical snake neurotoxic envenoming syndrome is characterized by descending, lower-motor neuron type flaccid paralysis due to disruption of neuromuscular transmission at the skeletal muscle NMJ [64, 185, 226, 229].

Snakebite neurotoxicity has been attributed to competitive inhibition of ACh binding to postsynaptic muscle nAChRs by α-3FNTxs or other α-neurotoxins; and/or PLA_2 related toxins that act presynaptically to cause a depletion of ACh synaptic vesicles and phospholipid hydrolysis resulting in irreversible destruction of the motor nerve terminal [185, 226]. Other contributing mechanisms have also been proposed for PLA_2 toxins (e.g. β-BgTx) that include an unclear role in binding to voltage-gated potassium channels [66], and interactions with muscle nAChRs [69-71].

Other neurotoxins like dendrotoxins and fasciculins in mamba venoms inhibit presynaptic voltage-gated potassium channels, and synaptic acetylcholinesterase, respectively; and together, cause excitatory effects and fasciculations [226]. κ-Neurotoxins (e.g. κ-BgTx) are inhibitors of α3 and α4 nAChRs and have little affinity for muscle nAChRs, and their clinical significance in snake envenomation is unclear since it is difficult for large peptides to breach the blood-brain barrier [64].

Antivenom is used in the treatment of snake envenomation [64, 226], and has been established to reverse postsynaptic neurotoxicity by neutralisation of α-3FNTxs in the intravascular compartment. While further neurotoxicity by PLA_2 can be prevented by antivenom, the damage already elicited to presynaptic nerve terminals is not reversible [64, 185, 230]. Silva et al. (2018) found that SC-α-3FNTxs, but not LC-α-3FNTxs, dissociated readily from human muscle nAChRs *in vitro*; unlike in rodent muscle where both were irreversible. They concluded that venoms dominated by SC-α-3FNTxs were unlikely to cause human paralysis [185]. Supporting this is clinical data showing that envenomation by snakes with a low relative abundance of LC-α-3FNTxs in their venoms, such as the sea snake *Hydrophis schistosus* (<14% LC-α-3FNTxs), Chinese cobra *Naja atra* (< 3% LC-α-3FNTxs) and Australian red-bellied black snake *Pseudechis porphyriacus* (no LC-α-3FNTxs detected), do not cause significant neuromuscular paralysis [185, 231].

Antivenom derived from polyclonal equine or ovine antibodies remains the mainstay of treatment for envenoming [232, 233]. However, this depends on snake species specificity, is high in production cost, and carries risks of acute or delayed allergic reactions [223, 226, 234]. Recombinant antivenom based on oligoclonal mixtures of human IgG antibodies may be vital for better and cost-effective snakebite envenoming therapies [232, 234]. The recently generated reference genome and transcriptome of the Indian cobra (*Naja naja*) has comprehensively catalogued the venom gland-specific toxin genes and identified a minimal set of 19 that constitute

the core venom toxins, which could facilitate the development of a safe and humanized antivenom directed against them [235].

An *in vitro* 'ligand-fishing' approach using the α7-AChBP chimera effectively captured LC-α-3FNTxs and offered significant *in vivo* protection when co-administered with a low, nonprotective dose of antivenom following cobra *Naja haje* envenomation in rodents [26]. Likewise, short HAPs with comparable affinity (IC_{50} ~ 2 nM) to intact nAChRs were also effective in capturing α-3FNTxs [49, 217], and capable of neutralizing α-BgTx *in vivo* in rodents [204]. This opens up their potential as treatment for envenomation [233, 236], where a single broad-spectrum nAChR mimic or combination of mimics targeting whole classes of neurotoxins could be used alone or adjunct with lower doses of antivenom, circumventing the concerns with polyvalent antivenom [26, 236].

16 | Conclusions and future directions

Emerging atomic-level structures of complexes between α-3FNTxs and nAChR models [57] have progressed to the 2.7 Å structure of α-BgTx bound to the intact *Torpedo* $(\alpha1)_2\beta1\gamma\delta$ nAChR, providing extraordinary insight into toxin-receptor interactions and gating mechanisms of the nAChR, and by extension, similar ligand-gated ion channel proteins [55]. The arsenal of snake toxins has also expanded exponentially, with the discovery of structurally and functionally novel classes of neurotoxins including NC-α-3FNTxs [126], dimeric α-3FNTxs [123, 140, 145], Ω-neurotoxins [135], drysdalins [130], αδ-neurotoxins [154] and ∑-neurotoxins [124].

The study of α-3FNTxs also provides clues to protein-protein interactions and evolution of protein scaffolds [3, 7], and the introduction of α-BgTx-binding pharmacophores into other membrane receptors, including AMPA and $GABA_B$, has enabled their characterization [50, 51]. Innovative high-throughput technologies have allowed the rapid discovery and screening of candidate

compounds, to envisage ambitious strategies for protein and genetic engineering and design of molecular probes, diagnostic markers and therapeutic leads from animal venoms.

Going back a full circle to when research into animal venoms was driven primarily by a need to treat envenomation, and in the context of snakebite being designated as a neglected tropical disease [225, 226], the future of treating envenomation neurotoxicity is looking beyond antivenom and at broad-spectrum nAChR mimics, including HAPs, to target whole classes of neurotoxins [26, 236].

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Conflicts of interest

The author declares no conflict of interests.

Figure legends

Figure 1

Proteins that modulate cholinergic function | (A) SC-α-3FNTx, **EbTx-b** (*Laticauda semifasciata*) (5EBX); **(B)** muscarinic AChR toxin, **MTx-1** (*Dendroaspis angusticeps*) (4DO8); **(C)** acetylcholinesterase inhibitor, **fasciculin-2** (*Dendroaspis angusticeps*) (1FAS); **(D)** presynaptic neurotoxic phospholipase A_2, **notexin** (*Notechis scutatus*) (1AE7); **(E)** LC-α-3FNTx, **α-BgTx** (*Bungarus multicinctus*) (1IK8); **(F)** neuronal nAChR antagonist, noncovalently-linked homodimeric **κ-BgTx** (*Bungarus multicinctus*) (1KBA); **(G)** covalently-linked heterodimeric NC-α-3FNTx, **irditoxin** (*Boiga irregularis*) (2H7Z); **(H)** NC-α-3FNTx, **candoxin** (*Bungarus candidus*) (1JGK); **(I)** NC-α-3FNTx and mAChR modulator, P33A mutant of **WTX** (*Naja kaouthia*) (1JGK); **(J)** ly6/UPAR related protein (soluble form), **Lynx-1** (*Homo sapiens*) (2L03); and, **(K)** secreted ly6/UPAR related protein, **SLURP-1** (*Homo sapiens*) (2MUO). All proteins shown except J and K, are derived from snake venom. All are 3FPs, except D. Proteins E through to K have an additional 5th disulfide bridge in their structure, with G, H, I, J and K conforming to the non-conventional 3FP scaffold where the 5th bond is in Loop I. All proteins act on muscle nAChRs except C, F, J and K; proteins E, F, H, I, J and K act on neuronal nAChRs; and, B and I act on mAChRs. The structures are α-carbon solid ribbon representations and presented with the aid of Weblab ViewerLite v3.2 (Accelrys Inc.). Disulfide bridges depicted in red, β-sheets in blue and α-helices in red. The species from which each protein was isolated and the respective PDB accession number for the toxin structure are indicated in parentheses. Loops I, II and III, as well as the C-terminal tail as 'C' are indicated. The toxins are not shown to scale.

Figure 2

Amino acid sequences of three-finger neurotoxins that interact with nicotinic acetylcholine receptors | The cysteine residues are bolded in red, and disulfide linkages are shown. The segments contributing to the three loops are shaded in grey. The number of amino acid residues in each sequence is indicated at the end of the respective sequence. The species names are as follows for **SC-α-3FNTxs**: erabutoxin b (*Laticauda semifasciata*), toxin-α (*Naja nigricollis*); **LC-α-3FNTxs**: α-bungarotoxin (*Bungarus multicinctus*), α-cobratoxin (*Naja kaouthia*); **NC-α-3FNTxs**: candoxin (*Bungarus candidus*), WTX (*Naja kaouthia*); **neuronal 3FNTxs**: κ-bungarotoxin (*Bungarus multicinctus*), κ-falvitoxin (*Bungarus flaviceps*); **dimeric 3FNTxs**: chains A and B of covalently linked heterodimeric irditoxin (*Boiga irregularis*); non-covalently linked homodimeric haditoxin (*Ophiophagus hannah*); non-covalently linked homodimeric fulditoxin (*Micrurus fulvius*); **atypical 3FNTxs**: colubrid NC-α-3FNTxs denmotoxin (*Boiga dendrophila*) with a longer N-terminus; αδ-Bungarotoxin-1 (*Bungarus candidus*) with higher selectivity for the α-δ binding interface of muscle nAChRs; drysdalin (*Drysdalia coronoides*) with a longer C-terminus; LSIII (*Laticauda semifasciata*) a LC-α-3FNTx with an unusually short C-terminal tail; Lc-b (*Laticauda colubrina*) a LC-α-3FNTx without the 5th disulfide bridge; Australian elapid Type III 3FNTx (*Pseudonaja textilis*) and Ω-Neurotoxin Oh-9 (*Ophiophagus hannah*) which lack all conserved canonical amino acid residues for nAChR interaction. The amino acid residues experimentally found to be critical for nAChR binding in SC-α-3FNTx, EbTx-a [42, 201, 211], and LC-α-3FNTxs, α-CbTx [134, 210] and α-BgTx [199, 202, 214] are indicated as follows: the common core of amino acid residues canonical for both SC-α-3FNTxs and LC-α-3FNTxs to bind to muscle nAChRs are **bolded in blue**; other specific residues experimentally found to contribute to binding to muscle nAChRs in EbTx-a (**bolded in green**); and α-CbTx or α-BgTx (**bolded in fuchsia**); are also shown;

where the specific residue also contributed to binding to α7 nAChRs, the **bolded residue is also underlined**; the key residues in WTX for interaction with muscle nAChRs are **bolded in black**. The presence of identical amino acid residues in homologous positions in other 3FNTx sequences is denoted in the respective colors (blue, green and fuchsia) but not bolded. The Cys residues contributing to the formation of covalent dimeric linkages are **boxed in red**. The PDB accession numbers and total amino acid residues in each toxin are indicated at the beginning and end of their sequence. *α-cobratoxin and κ-bungarotoxin are also covalently linked or non-covalently linked homodimers, respectively. #Fulditoxin is also an atypical 3FNTx as it lacks all conserved canonical amino acid residues for interaction with muscle or α7 nAChRs and is classed as ∑-neurotoxins.

Figure 3

The structure and subtypes of nicotinic acetylcholine receptors. **(A, B)** The 2.7 Å resolution cryo-electron microscopic structure of the *Torpedo* $(\alpha 1)_2\beta\gamma\delta$ nAChR (PDB accession # 6UWZ), as (A) viewed from the top, along its five-fold axis; and (B) viewed perpendicular to its five-fold axis. The nAChR structure, in its closed nonconducting state, was obtained in complex with α-BgTx bound at the α1-γ and α1-δ interfaces (*see Figure 5G*), which have been omitted for clarity. **(C)** Schematic diagram of the muscle or *Torpedo* nAChR of the stoichiometry $(\alpha 1)_2\beta 1\gamma\delta$ (with the γ subunit removed for clarity). Each subunit polypeptide is composed of four helical transmembrane domains (MI to MIV shown as brown α-helices) and a large N-terminal extracellular domain. **(E)** Classification of nAChRs as muscle or neuronal subtypes, based on the pentameric assembly of homologous subunits α1 to α10, β1 to β4, δ, γ and ε subunits. Orthosteric agonist binding sites are located in the extra-cellular domain, at ligand-binding interfaces which are uniquely defined by the subunit type that contributes to the primary (an α-subunit) (denoted by + symbol) or complementary (α- or non-α- subunit) surfaces. The muscle subtype is of the stoichiometry

α1β1δα1γ (clockwise) in the fetus, with the γ subunit replaced by ε in the adult form. The α1-δ interface is identical in both forms, but the second interface distinct depending on the complimentary subunit (α1-γ or α1-ε). The neuronal α7 and α9 nAChRs are homopentameric, with five identical ligand-binding interfaces between their respective subunits. All other neuronal subtypes have two, except $(\alpha4)_3(\beta2)_2$ which has three, binding interfaces as shown.

Figure 4

Canonical amino acid residues identified by mutagenesis studies in short-chain (erabutoxin-a) and long-chain (α-cobratoxin) α-3FNTxs for binding to nicotinic acetylcholine receptors. **(A)** The crystal structures of EbTx-a (2.0 Å) (PDB code: 5EBX), and **(B)** α-CbTx (2.4 Å) (PDB code: 2CTX), showing the residues by which, they interact with the *Torpedo* $(\alpha1)_2\beta1\gamma\delta$ nAChR. The concave faces of the toxins which project the side chains of all the functionally important residues are shown. The residues constituting the *common binding core* of critical amino acids in both SC-α-3FNTxs and LC-α-3FNTxs are shown in red. The key residues specific for each class of toxin for binding to *Torpedo* nAChRs are shown in dark green. In EbTx-a, specific residues that have supplementary roles (whereby their mutations resulted in < 10-fold decrease in affinity) are shown in blue. **(C)** The residues by which α-CbTx interacts with the α7 nAChR are shown in its concave surface. The residues which are crucial for α-CbTx binding to both, α7 and *Torpedo* nAChRs are shown in red; whereas those specific only for binding to α7 nAChRs are shown in purple. Models are based on Tremeau et al. (1995) and Ducancel et al. (1996) for EbTx-a; and Antil et al. (1999) and Antil et al. (2000) for α-CbTx. **(D, E)** The crystal structure of α-BgTx (0.8 Å) (PDB code: 1IK8) showing the residues by which it interacts with the α1 subunit. The concave face (D) which projects the side chains of key canonical residues shows the involvement of Loop I; and the convex face (G) shows the involvement of additional residues in Loop II and C-terminus.

The key ‘tetrad’ residues (bronze) and other interacting residues in Loop II (orange), Loop I (purple) and C-terminal tail (fuchsia) are shown, based on data from Dellisanti et al. (2007). Additional interacting residues shown in light purple (Pro10) and light pink (Lys70) are based on Huang et al. (2013). **(F)** The side view of EbTx-a (PDB code: 5EBX), showing the side chains of all the functionally important residues projecting from the concave surface. ViewerLite v5 (Accelrys Inc.) was utilized to show the CPK space-filling molecular representations for all except Panel (F) which is presented in solid ribbon format with the critical residues shown as sticks. The relative positions of Loops I, II and III, as well as the C-terminal tail as ‘C’ are indicated.

Figure 5

Canonical amino acid residues in long-chain three-finger α-neurotoxins for binding to nicotinic acetylcholine receptors identified by structural studies. **(A, B)** Overall 3.51 Å crystal structure of five α-BgTx molecules (in brown) in complex with the pentameric ligand-binding domain of an α7 nAChR-AChBP chimera (in grey) (PDB code: 4HQP) showing **(A)** the top view along the five-fold axis of the intact chimera, and; **(B)** side view perpendicular to the axis of the chimera (with toxin molecules 2, 3 and 5, and the chimera’s subunit 4 hidden for clarity). The chimera’s five subunits are numbered 1 to 5 in (A), and subunits 1 and 2 are in the foreground in (B). Loop C of each principal subunit of the chimera is highlighted in pink, and the key Tyr184 residue (*equivalent to Tyr190* in (D, E)) is shown in red (in all except subunit 2). In (A) and (B), the three canonical amino acid residues Arg36, Phe32 and Asp30 of the toxin that contribute to the *interacting tetrad* (together with Tyr184 of Loop C) are highlighted in bronze (in all except toxin 1), while additional residues of the toxin that contribute to binding are shown in beige (in toxins 2 and 5). The interacting residues are shown in CPK representation for toxins 4 and 5, and in ball-and-stick representation for toxins 2 and 3. **(C, D, E)** Overall 1.94 Å crystal structure of α-BgTx

(in brown) in complex with the ligand-binding domain of a single mouse α1 nAChR subunit (in grey) (PDB code: 2QC1) showing the side view perpendicular to the axis of the subunit. **(C)** Shows the interlocking of Loop C of the α1 subunit (pink) with Loop I, Loop II and C-terminal tail of the toxin; with the α1 subunit Loops A (blue) and B (green) also highlighted. **(D)** Enlarged view of (C) showing the interaction of the three toxin residues (bronze and beige) with Tyr190 (red) (*equivalent to Tyr184* in (A, B)) from the α1 subunit Loop C (in pink), in CPK representation. Together, they form the core *tetrad of interaction*. **(E)** The same binding interface shown in (D), but with the additional interacting residues from α1 subunit's Loop A (blue), Loop B (green) and Loop C (pink); and from the toxin's Loop I (purple), Loop II (orange) and C-terminal tail (fuchsia). **(F, G)** The 2.7 Å resolution cryo-electron microscopic structure of the *Torpedo* $(\alpha 1)_2\beta\gamma\delta$ nAChR in complex with α-BgTx (PDB accession # 6UWZ). **(F)** Enlarged view of the α1-δ interface with the primary (α1, in pink) and complementary (δ, in green) surfaces shown. α-BgTx is shown in brown. The key residues from the α1 primary surface, mainly from Loop C, are shown in red, while additional interacting α1 residues are in pink. Interacting residues from the δ complementary surface from Loop F, are shown in green. From α-BgTx, the key residues (Arg36, Phe32) are indicated in bronze while additional residues from Loop I (purple), Loop II (orange) and C-terminal tail (fuchsia), as well as Loop III (blue), are also shown. **(G)** The intact structure viewed from the top, along its five-fold axis; α-BgTx is bound at each of the α1-γ and α1-δ interfaces and stabilises the nAChR in a closed nonconducting state. In panels E and F, the protein chains are shown in line ribbon format, residues in stick format, and amino acids labelled in the single-letter code and position. In all other panels, protein chains are in solid ribbon format and the three-letter amino acid code and position is used to identify residues.

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