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Teaser This review presents the state-of-the-art for biological toxins targeting postsynaptic mechanisms of neurotransmission at the neuromuscular junction. It discusses emerging evidence for the therapeutic use of these biomolecules in the treatment of neuromuscular transmission disorders.

Neurobiology and therapeutic utility of neurotoxins targeting postsynaptic mechanisms of neuromuscular transmission

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The neuromuscular junction (NMJ) is the principal site for the translation of motor neurochemical signals to muscle activity. Therefore, the release and sensing machinery of acetylcholine (ACh) along with muscle contraction are two of the main targets of natural toxins and pathogens, causing paralysis. Given pharmacology and medical advances, the active ingredients of toxins that target postsynaptic mechanisms have become of major interest, showing promise as drug leads. Herein, we review key facets of prevalent toxins modulating the mechanisms of ACh sensing and generation of the postsynaptic response, with muscle contraction. We consider the correlation between their outstanding selectivity and potency plus effects on motor function, and discuss emerging data advocating their usage for the development of therapies alleviating neuromuscular dysfunction.

Introduction

The NMJ links spinal cord motor neurons to skeletal muscles via a specialized connection, where electrochemical nerve signals are transmitted into the contraction of striated muscles. This process is enabled predominantly via the release of ACh, which activates postsynaptic receptors, driving muscle contraction [1]. Structurally, the NMJ comprises three key constituents: (i) presynaptic nerve terminal; (ii) synaptic cleft; and (iii) postsynaptic receptive elements of the striated muscle (Fig. 1). The presynaptic motor nerve terminal represents the biogenesis site for synaptic vesicles and their loading with ACh followed by priming for fusion with the presynaptic membrane. Upon depolarization of nerve terminals, the influx of Ca^{2+} via voltage-gated channels triggers a complex sequence of molecular events, driving fusion of synaptic vesicles with the

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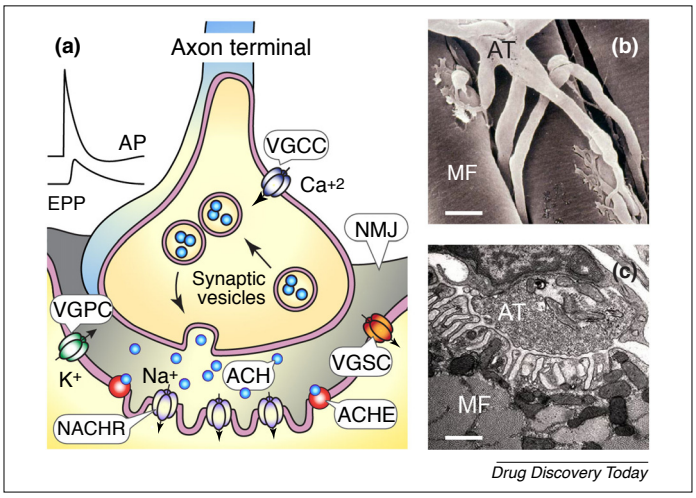


FIGURE 1

The neuromuscular junction (NMJ) as a site of transmission of neuronal electrochemical signals into muscle contraction. **(a)** Schematic of the neuromuscular synapse connecting axon terminal with a muscle fiber. The arrival of action potentials (AP) to the nerve terminal activates voltage-gated calcium currents (VGCC), which triggers the release of acetylcholine (ACH) into the synaptic cleft of the NMJ. Binding of ACH to postsynaptic nicotinic ACH receptors (NACHR) activates transmembrane voltage-gated sodium and potassium currents (VGSC and VGPC) leading to activation of end plate potentials (EPPs) with a muscle contraction. **(b)** Scanning electron microscopy image of the NMJ. (c) High-power transmission electron microscopy image of the NMJ. Scale bars: 2 μm (b) and 400 nm (c). Abbreviations: AT, axon terminal; MF, muscle fiber. Adapted from [195] (with permission) and www.anatomybox.com/neuromuscular-junction-tem. Courtesy of Dr. Wacker-Schröder (c).

surface membrane and ACh release into the synaptic cleft. Therein, small quantities of released ACh bind to receptors and activate elementary postsynaptic potentials, which then depolarize motor end plates, causing regenerative excitation waves and contraction of muscle fibers [2–4]. This complex and multistep reaction is followed by the rapid hydrolysis of released ACh by acetylcholinesterase (AChE) into choline and acetate [5,6], with reuptake from the NMJ and usage within a new activity cycle.

The core constituent of the postsynaptic receptive element at the NMJ is the end plate membrane, a specialized structure folded into multiple crests, which hold clusters of densely packed nicotinic ACh receptors (nAChR) [7]. It is the activation of these receptors that enables the influx of Ca^{2+} and Na^{+} into myocytes, driving depolarization of the junctional membrane and spreading excitation waves, leading to muscle contraction [8–11]. As a fundamental neurobiological process, synaptic transmission at NMJs has evolved to enable a range of motor activity [12]. Therefore, it is unsurprising that the NMJ is one of the most favored entry sites targeted by a range of neurotoxins and pathogens, specialized for causing synaptic transmission blockade with paralysis.

Research into the mechanisms and processes involved in neuromuscular paralysis by toxins has revealed four major junctures of high vulnerability: (i) the neurosecretory apparatus at presynaptic terminals; (ii) the postsynaptic nAChR; (iii) the machinery of ACh clearance from the synaptic cleft; and (iv) the ion channels driving the regenerative response at the postsynaptic site. The effects of neurotoxins on the presynaptic secretory apparatus and their action mechanisms have been the topic of numerous reviews, including analysis of their therapeutic utility [12–16]. Here, we consider research advances in the biology and medical translation of toxins that interfere with ACh activity within the synaptic cleft and at the postsynaptic aspect of NMJs. Such a systematic analysis will enhance understanding of basic mechanisms underlying their injurious effects and their promote potential utility to improve the management and treatment of neurological conditions related to abnormalities of synaptic transmission in the periphery and central nervous systems.

Reversible antagonists of postsynaptic nAChR: curare and curaremimetics

The largest and best-studied toxin group targeting nAChR at the NMJ is represented by the curare family, named after the plants of Central and South America, from which active ingredients were first extracted. Curare toxins belong to the class of bisbenzylisoquinoline alkaloids, which is a collection of (~400) natural products. Although bisbenzylisoquinoline alkaloids in general exhibit a variety of well-characterized biological activities, little is known about the pharmacological properties of other 'curare-like' alkaloids [17]. Traditionally, three different groups of curare products have been distinguished: (i) tube or bamboo curare (so-named because of its presence in bamboo tubes; known also as D-tubocurarine); (2) pot curare (so-named because of its storage in terracotta pots), includes protocurarine, protocurine, and protocuridine as main alkaloids, ranging from highly toxic to moderately toxic and nontoxic, respectively; and (3) calabash (packed by Amazonian hunters into hollow gourds) with the main toxin known as C-curarine I [18,19]. The history of the discovery of these toxins has been reviewed elsewhere [20–22] and expedited the identification of the first transmitter, ACh, and one of its receptors, nAChR. From all known variants, tubocurarine from *Chondrodendron tomentosum* is the most potent and best characterized, being widely used in clinical and veterinary practices since the 1940s as a potent muscle relaxant (Fig. 2a,b). Similar to most biological toxins, the effects of curare and curaremimetics involve a multistep process, entailing their entry into the circulation and spread to the site of toxicity, followed by penetration into the synaptic junction and the binding to clusters of nAChRs enriched in the sarcolemma (~10 000/μm^2), enabling competitive blockade of neuromuscular transmission [23]. The effect of tubocurarine is fully reversible, unlike irreversible nAChR antagonists (e.g., α-bungarotoxins). The reversibility of the effect is primarily because tubocurarine binding to nAChR is transient, followed by release from the nAChR binding site, a process crucial for full recovery of the paralyzed NMJ within several hours [24]. The latter is essential for developing antidotes countering the paralytic effect of curare, including cholinesterase inhibitors (e.g., pyridostigmine or neostigmine), which increase the level of ACh in the synaptic cleft of peripheral and central synapses.

At the molecular level, the inhibitory effect of tubocurarine is determined by its highly potent interaction with nAChR. As a member of the Cys-loop superfamily of ligand-gated ion channels, nAChR comprises five subunits (two α1, ε, δ, and β1), with binding sites localized at the interface of α1-ε and α1- δ [23,25] (Fig. 2c,d). ACh binding to the nAChR is a cooperative process, involving the interaction of at least two ACh molecules with the receptor sites [23]. The latter appears to be highly sensitive to the membrane lipid microenvironment, with optimal interactions requiring cholesterol molecules. As a typical benzylisoquinoline

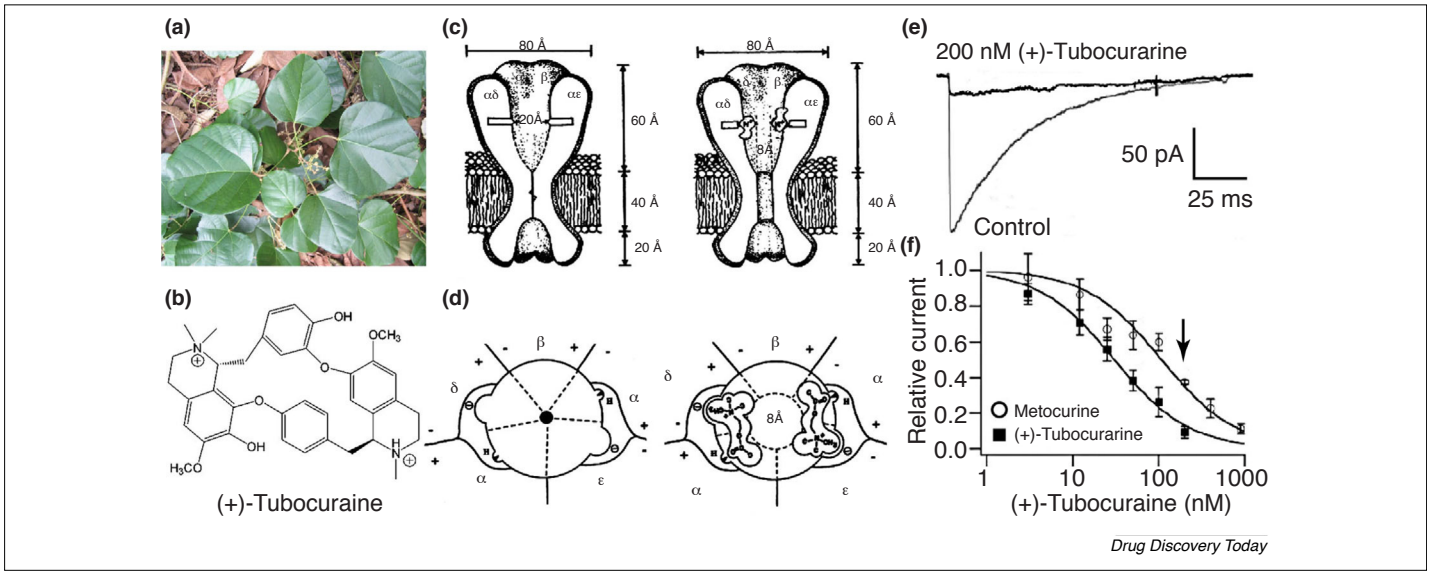


FIGURE 2

Blockade of neuromuscular junction (NMJ) transmission by curare. **(a)** Tubocurarine-producing *Chondrodendron tomentosum*. **(b)** Chemical structure of (+)-tubocurarine, which has been used over many decades as the main adjunct for clinical anesthesia. **(c,d)** Schematic representation of the fetal muscle subtype nicotinic acetylcholine (ACh) receptor (nAChR) demonstrating the receptor embedding in the membrane, with ACh binding to the channel pore in an open state. **(e)** Antagonising nAChR currents by (+)-tubocurarine. An example from an experiment with the use of ACh (300 μM) to activate membrane currents. (+)-Tubocurarine (200 nM) blocks the peak current. (f) (+)-Tubocurarine and metocurine compete for the antagonist binding site at the αε-subunit interface. The 3.5-fold shift in the apparent IC_{50} of (+)-tubocurarine when 170 nM metocurine (3× the IC50 of metocurine) is present, is consistent with competition for a single binding site. Adapted from sm.com.pk (a; with permission) and [27] (b). Adapted, with permission from Liu and Dilger [224] (e,f).

derivative, tubocurarine contains two positively charged nitrogen atoms protonated into a tertiary amine. Detailed atomic resolution analysis showed that tubocurarine is a monoquaternary rather than biquaternary alkaloid, as was previously believed [17]. In-depth research and molecular modeling studies showed that the distribution of charges and steric arrangements within tubocurarine are crucial for nAChR selectivity with a maintained interonium distance of ~1–1.4 nm. The latter appears to be key for the potency of tubocurarine at NMJs. Although the role of a second charged nitrogen in tertiary amine remains undecided, emerging evidence points to its enhancing effects on the binding process facilitating neurotransmission blockade [26]. Autoradiographic studies with labeled α-bungarotoxin showed that the two charges at the toxin center prevent each molecule bridging more than one nAChR or two α-subunits of the same receptor. The latter is largely determined by the distances between the ACh binding sites on the α-subunits, which are >1–1.4 nm [23]. It emerged that, for nAChR antagonists such as tubocurarine, a second charged center is necessary to ensure synergistic effects with an accessory anionic site that is not involved in ACh binding [27].

The effective and highly selective interaction of curare with the nAChR at the NMJ is key for its variety of clinical and veterinary usage. Nevertheless, numerous questions remain related to its action mechanisms. Much research is needed to define the molecular basis for its selectivity for nAChR in striated muscles, with advantageous pharmacokinetic and pharmacodynamic properties, which include rapid onset of paralysis, extended duration and full functional recovery, as well as fast clearance (Fig. 2e,f). Elucidating these important questions involves complex approaches with the use of a range of models, from *in silico* analysis of the receptor–ligand interactions to verifying studies in cell cultures as well as *ex vivo* NMJ preparations and whole animal and human studies. Since the first pioneering discoveries in 1912 using 'curarine' as an adjunct to anesthesia, a partially purified material from calabash curare has also been used as a potent surgical anesthetic [28], leading to the development of an array of pharmacological analogs with antinociceptive effects. Amongst these, pancuronium presents the most recognized example of rational drug design, although compounds such as atracurium are also widely utilized, because of their breakdown resulting in the production of inactive metabolites through a Hofmann elimination reaction [23,29,30]. The latter is especially useful in patients with kidney, liver or multiorgan failure, rendering functional recovery free of toxic byproducts and adverse effects. Another effective curare-based compound widely used as a potent anesthetic is vecuronium, which combines antinociceptive and myorelaxant actions, without significant adverse effects [31–33]. With the rapid onset of action, rocuronium is a favorable choice for use in patients at risk of gastric content aspiration.

Overall, combining muscle relaxant and anesthetic effects, curare mimetics have been considered over many decades as one of the best examples of toxin-derived drug leads. Following the arrival of modern muscle relaxants, independent control of anesthetic and relaxant effects has become possible, ensuring better and safer general anesthesia, independently from the relaxation of muscles, enabling more favorable conditions for surgery and pain management.

Three-finger toxins targeting neuromuscular transmission: α-neurotoxins

α-Neurotoxins represent active ingredients of elapid snake venoms causing acute and extensive muscular paralysis, which, if untreated, can lead to respiratory failure and death because of asphyxia [34]. Chang and Lee were first to isolate three types of

neurotoxin from the venom of krait *Bungarus multicinctus*: α-bungarotoxin, β-bungarotoxin, and γ-bungarotoxin, which were separated according to their electrophoretic mobility [35]. Using a chick biventer cervicitis nerve-muscle (CBCNM) preparation, it was later shown that α-bungarotoxin inhibited nerve-mediated muscle twitches by abolishing the contractile responses to ACh, implying a postsynaptic action site, unlike β- and γ-bungarotoxin, which acted presynaptically. After identification and purification, α-bungarotoxin became the tool of choice for the isolation and extensive characterization of nAChRs [36], which are currently the best-studied ionotropic receptor type [37–39].

Since pioneering research into nAChRs with the use of α-bungarotoxin, a large group of snake neurotoxins with postsynaptic action at skeletal muscles has been identified, which are categorized under the collective term 'α-neurotoxins' [40] (Fig. 3a). These represent a family of nonenzymatic proteins comprising 60–74

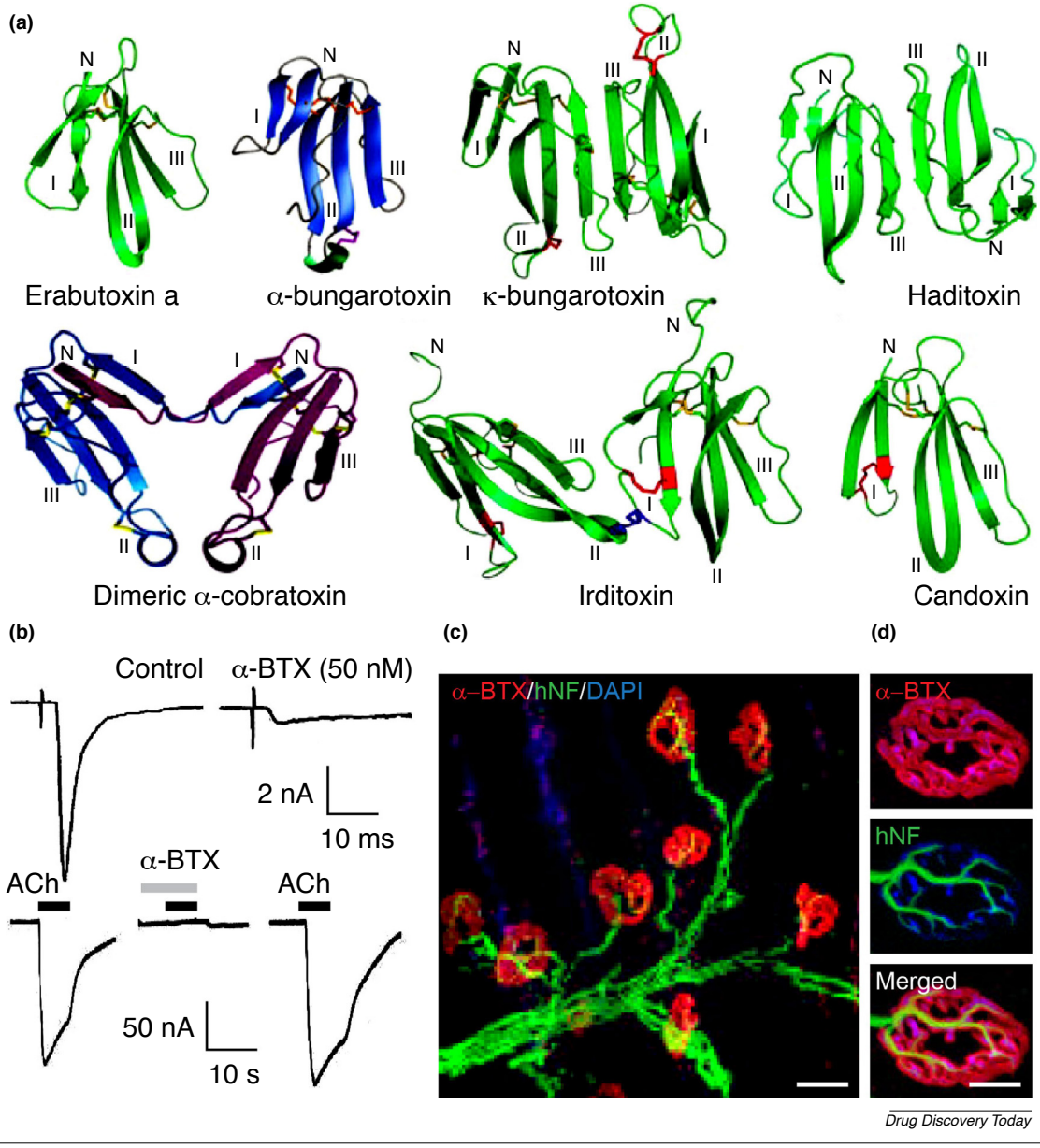


FIGURE 3

α-Bungarotoxin as a member of the three-finger toxin (3FTX) family: structure, effects, and utility. **(a)** 3D structures of 3FTXs that block nicotinic acetylcholine receptors (nAChRs). The loops ('fingers') are marked with Roman numbers I–III; 'N' indicates the N termini. Two monomers are shown in blue and magenta; 'nonconventional' disulfides in loops I are colored red and the disulfide bond between the monomers is indicated in blue. **(b)** Representative examples of the blockade of nACh currents by α-bungarotoxin at the central synapse and neuromuscular junction (NMJ; top and bottom traces, respectively). Current traces showing evoked responses before (control) and after 3-min incubation with a 50 nM application of α-bungarotoxin in a ciliary ganglion. Antagonism of Dα5 nAChRs expressed in oocytes by α-bungarotoxin (bottom). Representative responses to acetylcholine (100 μM; black bar) are shown (left), together with neuromuscular transmission blockade after 10-min pre-incubation with α-bungarotoxin (100 nM; gray bar) (middle). Recovery from the α-bungarotoxin blockade after 10 min is also illustrated (right). **(c)** Labeling NMJ of triceps brachii muscles of mice with neurofilament-specific antibodies (green) and α-bungarotoxin (red) with **(d)** higher power image of the end plate shown after color unmixing and merging. Scale bars: 6 and 2 μm (c,d, respectively). Adapted from [40] (a; with modifications), [196,197] (b; with permission), and [198] (c,d; with permission).

TABLE 1

Nomenclature and properties of α-neurotoxins isolated from snake venoms

Neurotoxin	Species	Molecular mass (Da)	Reversibility	Refs
Long-chain α-neurotoxins from elapid venoms				
α-Bungarotoxin	*Bungarus multicinctus*	7983	Irreversible	[35]
α-Cobratoxin	*Naja kaoutia*	7820	Irreversible	[203]
α-Elapitoxin	*Acanthophis antarcticus*	8850	Pseudo-irreversible	[59]
Mikatoxin	*Micropechis ikaheka*	7775.6	Irreversible	[204]
Acantophin a	*A. antarcticus*	7155	ND	[205]
Acantophin d	*A. antarcticus*	8800	Irreversible	[206]
Pseudonajatoxin a	*Pseudonaja textilis*	7762	ND	[207]
Pseudonajatoxin b	*P. textilis*	12 280	Irreversible	[208]
Short-chain α-neurotoxins from elapid venoms				
Erabutoxin (a & b)	*Laticauda semifasciata*	6837/6868	Reversible	[58]
α-Oxitoxin 1	*Oxyuranus scutellatus canni*	6770	Reversible	[209]
α-Scutoxin 1	*Oxyuranus scutellatus scutellatus*	6781	Pseudo-irreversible	[209]
Papuantoxin-1	*Pseudechis papuanus*	6738	Pseudo-irreversible	[210]
Hostoxin-1	*Hoplocephalus stephensi*	6660	Poorly reversible	[211]
Oxilepitoxin-1	*Oxyuranus microlepidotus*	6789	Reversible	[212]
Taipan toxin (1 & 2)	*O. s. scutellatus*	6726/6781	ND	[213]
Acantoxin IVa	*Acanthophis* sp.	6815	Pseudo-irreversible	[214]
Pt-N1 & N2	*P. textilis*	6236/6345	ND	[215]
α-Neurotoxins from colubrid venoms				
Boigatoxin-A	*Boiga dendrophila*	8679	Pseudo-irreversible	[46]
Denmotoxin	*B. dendrophila*	8508	Irreversible	[47]
Rufoxin	*Rhamphiophis oxyrhynchus*	10,661	Partly reversible	[48]
Candoxin	*Boiga candidus*	7334.6	Reversible	[204]
α-Colubritoxin	*Coelognathus radiatus*	8498	Partly reversible	[41]
Dimeric α-neurotoxins from snake venoms				
k-Bungarotoxin	*B. multicinctus*	7313	Reversible	[40]
Dimeric α-Cobratoxin	*N. kaoutia*	15 640	Irreversible	[51]
Haditoxin	*Ophiophagus hannah*	16 250	Partly reversible	[50]
Irditoxin (A+B)	*Boiga irregularis*	8379+8677	Partly reversible	[52]

amino acids present in the venoms of elapid snakes (e.g., cobras, mambas, kraits, sea snakes, etc.) [41–43]. At present, >100 α-neurotoxin variants have been isolated and sequenced [44]. Based on structure and molecular identity, α-neurotoxins are classified into short-chain (60–62 amino acid residues and four disulfide bonds) and long-chain (66–74 amino acid residues, four disulfide bonds, and an additional disulfide bond in the tip of loop II) toxins [45] (Table 1). Several new α-neurotoxins, including boigatoxin-A [46], α-colubritoxin [41,42], denmotoxin [47], and rufoxin [48], have also been isolated from colubrid venoms. Typically, colubrid α-neurotoxins are larger than elapid short- and long-chain α-neurotoxins, ranging from 8.5 to 10.7 kDa [47,48]. They also show high homology in their N-terminal sequences, except for rufoxin [49]. In contrast to both short- and long-chain α-neurotoxins, colubrid α-neurotoxins do not contain a cysteine residue at position 3 and/or 4 and, therefore, cannot form a disulfide bridge at this residue [48]. Nonetheless, most evidence suggests a total of five disulfide bridges in other positions throughout the molecule [47,49]. Finally, there is a small group of dimeric α-neurotoxins, with the best-characterized representative κ-bungarotoxins [40], known to interact preferably with neuronal nAChRs. Haditoxin, which also belongs to homodimeric toxins from the venom of the king cobra (*Ophiophagus hannah*) lacks its intermolecular covalent bonds and comprises two short-chain peptides, known to act as an effective and selective blocker of muscle-type nAChR [50]. α-Cobratoxin from *Naja kaouthia* venom and irditoxin from *Boiga irregularis* are two other examples of dimeric neurotoxins, formed by covalently bound monomers. Interestingly, in α-cobratoxin, the dimer is formed by two molecules connected via two disulfide bonds with a lower activity against muscle-type nAChR [51], whereas in irditoxins, because of the presence of an additional cysteine residue, monomers are connected by a single disulfide bond [52]. Overall, because of a well-recognized tendency to fold as three β-stranded loops extending from a small hydrophobic core cross-linked by four conserved disulfide bridges, all these peptides are also known as 'three-finger toxins' (3FTXs) [53,54].

All members of the α-neurotoxin family share similar and relatively straightforward mechanisms of action, via targeting and binding postsynaptic nAChRs at the NMJ, causing neuromuscular paralysis [36,45,55]. α-Neurotoxin binding with the nAChR is a two-step process, resulting from an initial attachment to the cell membrane mediated through tight electrostatic interactions of toxins with the lipid bilayer, although some evidence for the direct interaction of toxin with nAChR has been also reported. As a result of the formation of hydrogen bonds between the polar head groups of the lipid bilayer of the membrane and hydrophilic, positively charged short-chain of the toxin, the precise positioning of the loop II of the neurotoxin is achieved for binding nAChR [56]. Once bound to the nAChR, α-neurotoxins prevent its interactions with ACh. Such an action mechanism qualifies α-neurotoxins as a curarimimetic; however, unlike the reversible effects of curare, α-neurotoxins show a range of interactions, including reversible, pseudo-irreversible, and irreversible interactions [49]. It is generally accepted that long-chain α-neurotoxins have irre-

versible effects compared with the reversible effects of short-chain α-neurotoxins. Given that both active binding sites of the nAChR need to be occupied by ACh to induce the opening of the ion channel function and cation influx, the occupation of one binding site by the α-neurotoxin disrupts the normal functions of the channel and blocks nAChR-mediated currents. The lack of a fifth disulfide bridge in short-chain α-neurotoxins limits their effects on muscle α1 nAChRs only, whereas long-chain α-neurotoxins can also bind to neuronal nAChR (α7 type) with relatively high affinity (Fig. 3b) [39,57,58].

Although snake venoms and their active ingredients have been extensively investigated for their potential usage as lead therapeutic compounds, currently there are no drugs on the market that have been derived from α-neurotoxins. Nevertheless, some short-chain and long-chain α-neurotoxins showing reversible effects *in vitro* (Table 1) have been suggested to present potential therapeutic value for the treatment of diseases associated with NMJ dysfunction [59]. As highly potent and selective agents targeting the NMJ, 3FTXs have contributed significantly towards our understanding of mechanisms of venom toxicity and neuromuscular paralysis. Likewise, research into the biology and mechanisms of these toxins has provided useful tools for deciphering the molecular events and neurophysiological processes unfolding at NMJs [60,61]. For example, understanding the role of α-bungarotoxin enabled the first isolation and characterization of nAChR, contributing towards unraveling mechanisms of neuromuscular diseases, such as myasthenia gravis [38,62]. Given their high selectivity for specific nAChR subunits, α-neurotoxins have also been utilized for mapping the expression of nAChRs in particular tissues or cell types, with identification of receptor subtypes, as well as for imaging their trafficking [63–65] (Fig. 3c,d). k-Bungarotoxin has also been used for deciphering molecular mechanisms of long-term potentiation and synaptic memory [66], whereas the three-finger muscarinic toxins (MT1 and MT2 from various African mamba venoms) selective for M4 muscarinic receptors (mainly found in the central nervous system) have been used for induction of retrograde amnesia and memory-deficit animal models [67]. Other α-neurotoxins, such as cobratoxin from Thailand cobra (*Naja kauothia*) has been considered as therapeutic candidates for the treatment of adrenomyeloneuropathy and multiple sclerosis [68], whereas α-cobratoxin has shown promise as a potential anticancer drug in non-small cell lung cancer [69].

Acetylcholinesterase inhibitors interfering with neuromuscular transmission: fasciculins

In addition to natural toxins inhibiting neurotransmission via targeting the ACh release machinery or postsynaptic receptors, some are capable of blocking neuromuscular transmission by hindering the mechanisms of ACh clearance after exocytosis, which leads to spastic paralysis. Among these, fasciculins are the best-characterized family, represented largely by three types of polypeptide: type 1 and 2 extracted from the venom of the eastern green mamba *Dendroaspis angusticeps* [70,71] and type 3 from the venoms of the black mamba, *Dendroaspis polylepis* [72] and the western green mamba, *Dendroaspis viridis* [73]. Comprising 61 amino acid residues with four disulfide bridges, fasciculins reveal structural features resembling those of short-chain neurotoxins and 3FTX family members [74].

Fasciculins were first purified from green mamba venom [75] and initially described as potent noncompetitive inhibitors of neuromuscular transmission, through binding to AChE at the NMJ, which is the only enzyme catalyzing the breakdown of ACh in the synaptic cleft [76]. This results in accumulation of ACh in the synaptic cleft of the NMJ with persistent activation of AChR, causing blockade of neuromuscular transmission. Once formed, the high-affinity fasciculin–AChE complex is slow to dissociate and, hence, leads to a persistent spastic state [77]. Given the omnipresence of AChE in vertebrate NMJ, fasciculins have ubiquitous effects, from zebrafish to humans (Fig. 4a).

Structurally and functionally, AChE is a representative of serine hydrolases of higher eukaryotes and belongs to the esterase family. The efficacy of AChE as a key regulator of ACh lifetime and activity at peripheral as well as central synapses depends on its enzymatic activity, as well as on its density and location relative to the receptors [78]. At the NMJ, AChE tetramers are specifically clustered in the basal lamina by collagen Q (ColQ) [5]) and are maintained at high density and stability ($t_{1/2} \approx 20$ days) [79,80]. In vertebrates, AChEs have been classified in several variants based on their quaternary structure and the number of glycoprotein catalytic subunits [81]. The most prevalent globular forms of AChE contain one, two, or four catalytic subunits, known as G1, G2, and G4, respectively. The second most-prevalent forms of AChE are characterized by the presence of a collagen-like tail associated with one, two, or three tetramers and, hence, are named A4, A8, and A12 [82]. Despite this diversity, all vertebrate AChEs are encoded by a single gene, with all molecular variants resulting from alternative mRNA splicing and some post-translational modifications [83]. The steric organization of AChE forms a deep (20 Å) and narrow (5 Å) cavity lined with 14 aromatic residues (Fig. 4d), with its active site located near the bottom of the cavity [84]. During breakdown, ACh first binds to the peripheral anionic site (PAS) of AChE, which is followed by funneling down the cavity to the active site energized by the interaction of its quaternary ammonium group with the aromatic rings of 14 aromatic amino acid residues lining the AChE cavity [85]. At this active site, which contains a Glu/His/Ser catalytic triad, upon interaction with ACh, the latter is oriented for hydrolysis by interactions between the catalytic anionic site of the enzyme and quaternary ammonium group of ACh. However, this geometry fails to explain the remarkably high rate of catalytic activity of the enzyme, which can reach a turnover rate of up to 10^4 s^{-1} [86].

The specific distribution of charges in AChE favors a large dipole moment in the cavity interface, which serves to attract the positively charged substrate ACh into the active site (Fig. 4b,c). This relatively strong electrochemical gradient extends along the whole length of the gorge, which is used to pull the substrate down into the cavity, once it has entered its mouth [87]. Given the weak hydration, ACh readily interacts with the aromatic residues Trp279 and Tyr70 (so-called π-cation interactions) at the top of the gorge, as well as Tyr121 and Phe330 at the bottleneck. Electrostatic calculations based on the Torpedo AChE structure and subsequent molecular dynamic simulation suggested that AChE also has a so-called 'back door', distinct from the cavity entrance, the transient opening of which would contribute to the high rate of traffic of substrates, products, and water into and out of the active center [88,89]. The opening of this putative molecular gate

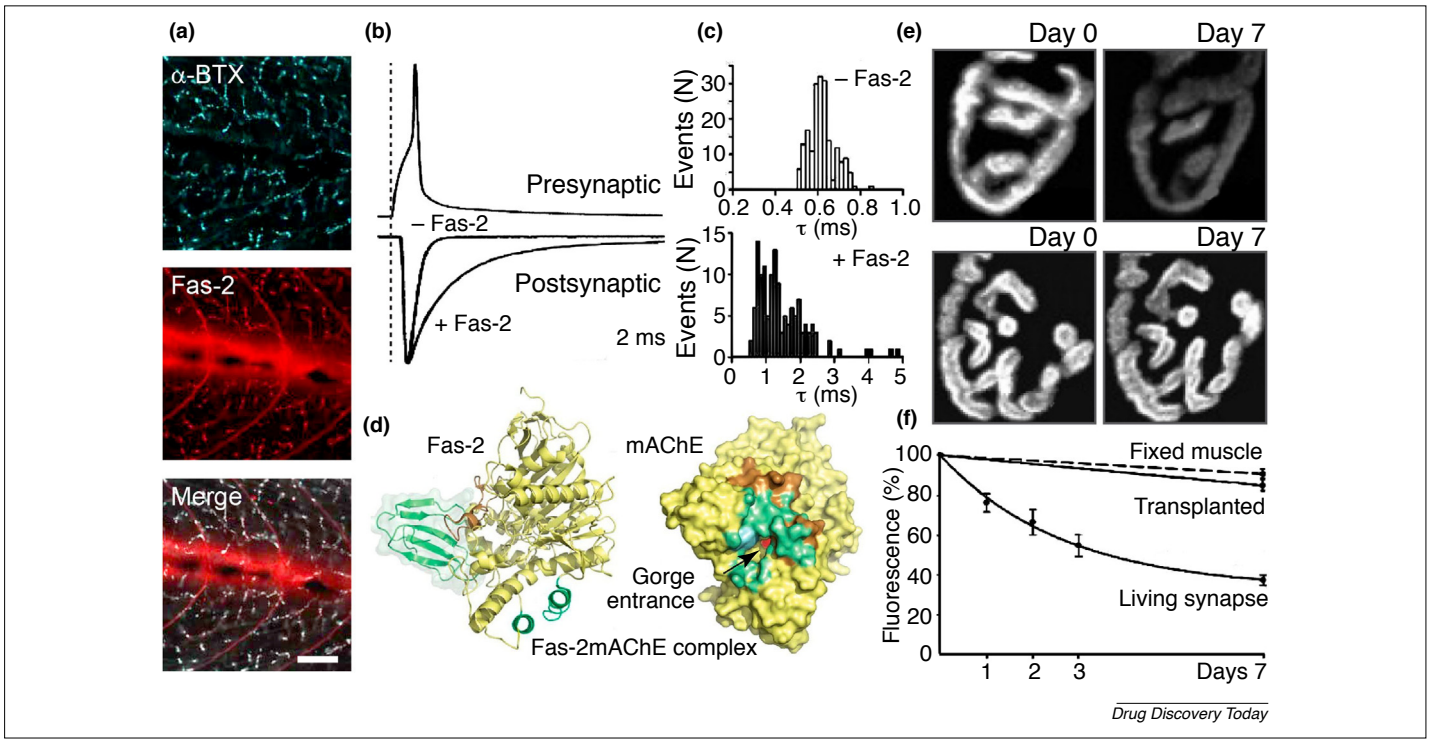


FIGURE 4

Fasciculins as regulators of acetylcholine esterase activity at the neuromuscular junction (NMJ). **(a)** Confocal microscopic images demonstrating colocalization of the postsynaptic marker α-bungarotoxin (α-BTX) and fascicullin 2 (Fas-2) in the zebrafish tail muscle. **(b)** The motor neuron action potential (top) and normalized end plate currents (EPCs) before and after treatment with Fas-2. Each trace represents the average of 10–30 events from a single recording trail at 50 mV membrane potential. **(c)** Comparisons of decay time constants of EPC kinetics in control (top) and acetylcholinesterase (AChE)-inhibited (right) muscle with Fas-2 (250 –300 nM). **(d)** The overall views of the crystal structure of the Fas-2–mAChE complex (left; accession code: 1KU6) and the buried interface at the mAChE surface, oriented 90° from each other (right). The mAChE subunit is displayed in yellow and Fas-2 in green [88]. **(e)** Examples of live and fixed NMJ labeled with fasciculin-2-Alexa594 and measured at two time points. **(f)** Graph summarizing results obtained over 7 days from all NMJs with approach shown in **(e)**. Each data point represents the mean percentage of fluorescence intensity, showing a gradual loss in living NMJ, proving that Fas-2 can be used as a probe for studying AChE dynamics at the NMJ. Adapted, with permission, from [199] (a-c). Adapted, with permission from Martinez-Pena y Valenzuela *et al.* [78] (e,f).

would involve displacement of the Trp-86 side chain located at the base of the active center, which separates the choline-binding site from the outside solvent, a conformational change that shifts the Trp-86 side chain and opens an alternative entry portal for the substrate. The latter is vital for AChE inhibition, because fasciculins bind the PAS and mechanically seal the cavity entrance [90,91], blocking its catalytic activity. As a result, any residual activity of AChE needs to be implemented through an alternative entry point. Moreover, electrostatic interactions between cationic residues on the interaction surface of fasciculins and anionic residues on the interaction surface of AChE appear to have an important role in the binding process [92]. Loop II in fasciculins differs from that in structurally related α-neurotoxins in having six positively charged amino acid residues. Fasciculins also differ from other 3FTXs in having Arg at position 11. The side chains of these conserved basic residues might be important for interactions with AChE. In addition, fasciculins also have another cluster of positive side chains on the opposite face of the molecule (Arg24, Arg37, Lys51, and Lys58 positions), which makes them basic molecules with a net charge of +4. Despite intense research, the mechanism of the fasciculin–AChE interaction remains open for debate, with crystal structure studies not yet revealing an open back door scenario, leaving open possibilities for alternative mechanisms of its interaction with ACh and breakdown [93,94].

Dense clustering of AChE tetramers in the basal lamina of the NMJ by ColQ [5] maintains its stable activity [79,80] and renders the tetramers not only effective in the breakdown of ACh, but also as reliable markers for mapping the NMJ as well as AChE-enriched elements at the basal lamina (Fig. 4e,f). The latter has been used as a highly specific biochemical tool for quantifying AChE distribution and density at the NMJ with radiolabeled fasciculin-2 [95], whereas fluorescent fasciculin-2 has been applied to label AChE enrichment in cultured muscle tissue [96], and in functioning synapses [97]. Complexes of Fas with mammalian AChEs display high affinities ($K_d = 10^{-11}$–10^{-12} M) [76,98,99], with tight binding between these two proteins attributed to factors such as: (i) large hydrophobic surface facilitating their effective interactions; (ii) formation of intermolecular hydrogen bonds; and (iii) remarkable surface complementarities [91].

On the functional side, the effective breakdown of ACh by AChE and its clearance makes fasciculins suitable for studying AChE dynamics at the NMJ and modulating synaptic transition at central cholinergic synaptic connections [100], with inhibition of AChE leading to several therapeutically relevant outcomes, depending on the physiological context and location. At the periphery, for instance, similar to the use of α-bungarotoxin for studying nicotinic receptors, fasciculins have been used for deciphering the structure and mechanism of action of AChE [73]. Low

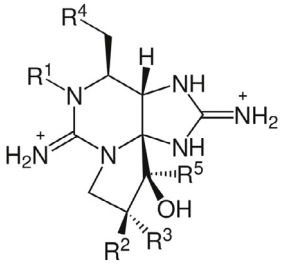
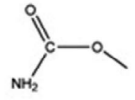
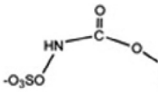
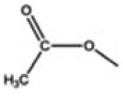
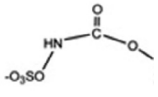
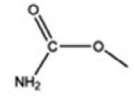
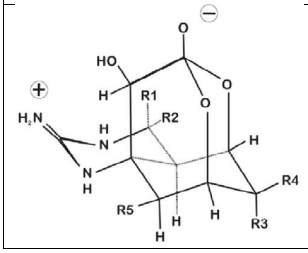
mobility and inability to diffuse far from the site of injection also render fasciculins suitable for studies in the central nervous system, using microinjections into specific brain regions, causing localized and long-lasting inhibitory effects [101]. Given that fasciculins can be labeled with I^{125}, they have also been applied as probes for AChE in quantitative autoradiography [73,95]. Likewise, in developmental studies, fasciculins have been utilized to investigate the role of AChE in governing the outgrowth of neurites from nerve cells, an effect attributable to the hydrolytic action of enzymes on ACh [102] as well as its role as an adhesion factor [103]. Similarly, in neuroblastoma cells transfected to express AChE, treatment with fasciculins caused a significant decrease in both the number of neurites and their length [104]. Even though the poor penetration of fasciculins to the brain and spinal cord impose major limits for their utility as enhancers of cholinergic drive in central synapses, the use of different types of natural AChE blocker for countering cognitive deficit and memory loss in Alzheimer's disease has been widely discussed [105]. Hence, the possible usage of fasciculins for these and several other medical applications is currently under consideration.

Blockers of regenerative responses at the neuromuscular junction

The potent paralytic effects of fish toxins were recognized by ancient Egyptians, Greeks, and Chinese [106]. In Caribbean voodoo rituals, puffer fish extracts were used for zombification [107], whereas native populations of the Pacific Northwest of America documented periods of 'shiny water', a time of year when shellfish consumption was especially dangerous [108]. Research over the second half of the past century attributed these effects to guanidinium toxins, comprising saxitoxin (STX) and tetrodotoxin (TTX), natural alkaloids present in high amounts in puffer fish extracts. With an exceptional high affinity for binding to voltage-gated sodium channels (Na_v) and blockade of Na^+ influx at pre- and postsynaptic elements of NMJs, these toxins impede the generation and propagation of action potentials, causing muscular

TABLE 2

Major natural analogs of STX and TTX[a]

STX	Analog	R1	R2	R3	R4	R5
	STX - saxitoxin	H	H	H		OH
	NEO - neosaxitoxin	OH	H	H		OH
	GTX1- gonyautoxins	OH	H	OSO_3^-		OH
	GTX2	H	H	OSO_3^-		OH
	GTX3	H	OSO_3^-	H		OH
	GTX4	OH	OSO_3^-	H		OH
	B1	H	H	H		OH
	B2	OH	H	H		OH
	C1	H	H	OSO_3^-		OH
	C2	H	OSO_3^-	H		OH
	C3	OH	H	OSO_3^-		OH
	C4	OH	OSO_3^-	H		OH
	dcSTX – decarbamoyl STX	H	H	H		OH
	dcNEO – decarbamoyl NEO	OH	H	H		OH
	dcGTX1 – decarbamoyl GTX	OH	H	OSO_3^-		OH
	dcGTX2	H	H	OSO_3^-		OH
	dcGTX3	H	OSO_3^-	H		OH
	dcGTX4	OH	OSO_3^-	H		OH
	LWTX1 - lyngbyatoxins	H	H	OSO_3^-		H
	LWTX2	H	H	OSO_3^-		OH
	LWTX3	H	OSO_3^-	H		OH
	LWTX5	H	H	H		OH
	LWTX6	H	H	H		H
	LWTX4	H	H	H	H--	H
	M1	H	OH	H		OH
	M3	H	OH	OH		OH
	M2	H	OH	H		OH
	M4	H	OH	OH		OH
TTX	**Analog**	**R1**	**R2**	**R3**	**R4**	**R5**
	TTX	H	OH	OH	CH_2OH	OH
	4-epiTTX	OH	H	OH	CH_2OH	OH
	6-epiTTX	H	OH	CH_2OH	OH	OH
	11-deoxyTTX	H	OH	OH	CH_3	OH
	6,11-dideoxyTTX	H	OH	N	CH_3	OH
	11-oxoTTX	H	OH	OH	$CH(OH)_2$	OH
	11-norTTX-6(R)-ol	H	OH	H	OH	OH
	11-norTTX-6(S)-ol	H	OH	OH	H	OH
	Chiriquitoxin	H	OH	OH	$CH(OH)CH(NH_3^+)COO^-$	OH

LWTX 1-6 *from a genus of cyanobacteria Lyngbya;* **M-toxins** *from mussels Mytilus*

paralysis. Given their small (typically 200–600 Da) size and heat resistance, guanidinium toxins cannot be neutralized by cooking or boiling. Whereas TTX and its analogs bear one guanidinium group, STX carries two, which results in a major difference in their characteristics [109]. Nonetheless, both groups of toxin are highly potent, with the lethal dose for humans in the low milligram range, qualifying them among the most potent natural poisons (Table 2).

TTX is perhaps the best studied of all biological toxins. The main biological source of the TTX is the pufferfish (Fig. 5a,b), with high amounts also present in other aquatic animals (e.g., moon snails and blue-ringed octopuses). However, the source of TTX in brachiopods is a matter of debate, with available evidence suggesting that these animals accumulate TTX directly from marine bacteria that synthesize the toxin (e.g., *Vibrio, Pseudoalteromonas*, and *Pseudomonas*) [108]. With dual effects at the NMJ, TTX blocks voltage-activated Na^+ currents at nerve terminals and striatal muscles [110] through specific binding to the pore-opening site of the Na^+ channel, disabling its function and leading to action potential blockade [111]. The binding site for TTX is located in the outer vestibule of the α-subunit of the Na^+ channel, in a water-filled region formed by four P-loops of Na_V proteins (Fig. 5c,d) [112]. Complete or partial blockade of the channel are both mediated through binding to the anionic carboxylate groups of the D and E residues of the so-called 'DEKA selectivity filter', with 1:1 stoichiometry [113]. Such specific TTX-Na_V interactions have been instrumental in the identification and characterization of voltage-gated Na^+ channels and their role in the generation and propagation of action potentials in axons (Fig. 5e,f). Indeed, the selectivity of TTX for Na^+ channels had a key role in Nobel Prize-winning research led by Hodgkin and Huxley, which led to the deciphering of molecular mechanisms underlying the generation and propagation of action potentials [114]. The subsequent cloning and analysis of Na^+ channel structure and function enabled the discovery of a large number of genetic diseases, including periodic paralysis, cardiac arrhythmia, epilepsy, and chronic pain caused by Na_V channel dysfunctions [115]. Based on sensitivity to TTX, two distinct types of Na_V channel are distinguished: TTX sensitive and

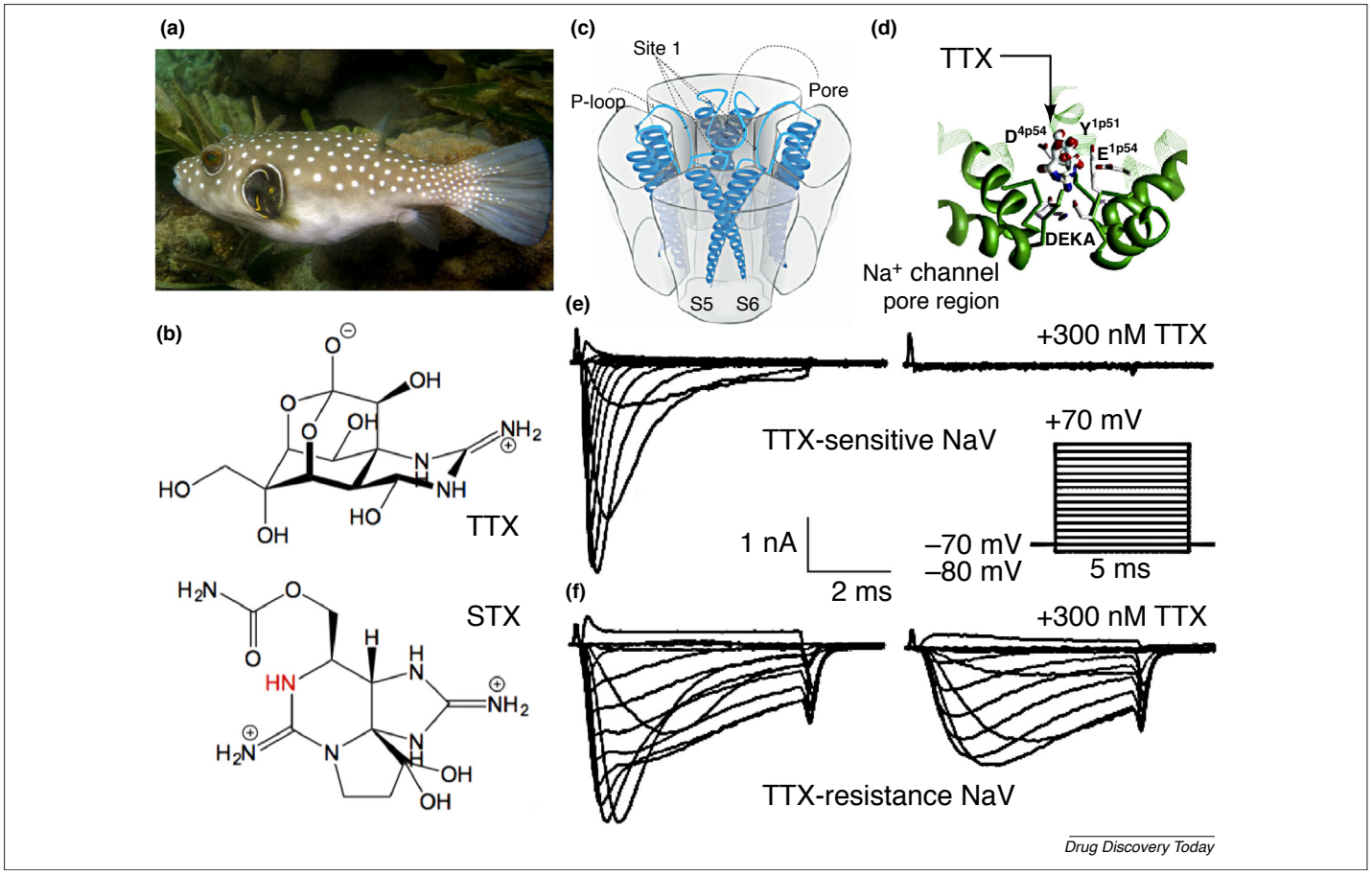


FIGURE 5

The biology and mode of action of tetrodotoxin. **(a)** Pufferfish *Takifugu niphobles,* one of the members of Tetradontiformes order carrying large amounts of tetrodotoxin. **(b)** Chemical structure of tetrodotoxin and saxitoxin (TTX and STX, respectively). **(c)** Schematic of the α-subunit of the voltage-gated Na^+ channel contains with four homologous repeats, with S5–S6 and the P-loop forming the pore of the channel and site 1, which forms the binding site for both STX and TTX. **(d)** Side view of the $Na_V1.4$ model with bound TTX. The model readily reproduces experimental data on TTX–channel interactions, including contacts with the selectivity-filter residues Asp-Glu-Lys-Ala (DEKA) and the outer carboxylates as well as cation-π interactions with Tyr^{1p51}. **(e,f)** Representative recordings of Na^+ currents. Endogenous TTX-sensitive Na^+ currents of ND7/23 cells in the absence or presence of 300 nM TTX, sufficient to block TTX-sensitive channels **(e)**. Recordings from ND7/23 cells transfected with TTX-resistant $Na_V1.8$ in the absence and presence of 300 nM TTX (left and right, respectively). Insets show stimulation protocols **(f)**. Adapted with permission from [113] (d) and [200] (e,f).

TABLE 3

The acute toxicity of guanidinium toxins in mice via various routes of administration

Toxin	Route of administration	LD_{50} (nmol/kg)	Refs
STX	i.v.	11–28	
	s.c.	43	[125]
	i.p.	26–33	
NEO	i.p.	8.9	
dcSTX	i.p.	35.4	[216]
GTX1/4	i.p.	14.6	
GTX1/3	i.p.	36.7	
STX	O/G	1190	[216]
	O/F	3200	
NEO	O/G	700	[216]
	O/F	1260	
dcSTX	O/G	2600	[216]
	O/F	8680	
GTX1/4	O/G	1610	[216]
	O/F	3420	
GTX1/3	O/G	2230	[216]
	O/F	5590	
TTX	i.v.	28	[217]
	i.p.	34	[218]
	s.c.	39–50	[219]
	i.g.	1668	[218]
11-deoxyTTX	O	727	[220]
	i.p.	231	

[a]i.g., intragastric; i.m., intramuscular; i.p., intraperitoneal; i.v., intravenous; o, oral; O/F, oral/feeding; O/G, oral/gavage; s.c., subcutaneous.

TTX resistant [116], with IC_{50} 5–15 nM (Na_v 1.1–1.4, 1.6, and 1.7), and low micromolar binding affinities (Na_v 1.5, 1.8, and 1.9), respectively. Importantly, cells expressing TTX-resistant Na^+ channels are located mainly in the myocardium, whereas TTX-sensitive Na^+ channels are found throughout the nervous system and other excitable tissues in vertebrates [117–119].

Similar to TTX, STX poisoning can be caused by shellfish or by toxin-contaminated seafood. Every year, >2000 cases of paralytic shellfish poisoning occur around the world, with a mean fatality rate of ~15% (Table 3). The main natural sources of STXs are various species of bivalve shellfish, feeding on toxic dinoflagellates as well as crustaceans and gastropods. In the sea, STXs are produced by members of three genera of free-living dinoflagellates, which include more than ten species of *Alexandrium*, and single species of *Pyrodinium* (*P. bahamense* var. *bahamense*) and *Gymnodinium* (*G. catenatum*) [120,121]. Remarkably, some species of freshwater filamentous cyanobacteria [122,123] from the orders *Oscillatoriales* and *Nostocales* are also capable of producing STX or its analogs. In humans, STX poisoning appears ~30 min after consumption of sufficient amounts of toxic food, which causes characteristic tingling of the tongue and lips followed by a sensation of floating, headache, vomiting, muscle weakness, and ataxia followed by deterioration of speech and disruption of eye movement and swallowing. Of note, consciousness in most cases remains unaffected. With the intake of high doses (~1–4 mg), lethality can occur because of the arrest of breathing caused by the paralysis of respiratory muscles [124,125]. Similar to TTX, STX comprises a central carbon atom and three nitrogen atoms with a positive charge at physiological pH, which confer the binding capacity to site 1 of Na_V, thereby causing partial or complete blockade of the Na^+ current (Fig. 5b). However, unlike TTX, STX analogs also interact with Ca_V and K_V ion channels in addition to Na_V, albeit with different potency. Interestingly, STX also blocks K_V channels through changes in channel gating, but not by pore blockade, with up to four toxin molecules capable of binding simultaneously to the channel [126,127]. In contrast to K_V and Na_V channels, the action of STX on Ca_V channels in ventricular myocytes is mediated via a reduction in the Ca^{2+} current [118].

As noted earlier, TTX and STX poisoning lead to the loss of sensation with extensive paralysis of voluntary muscles, including the diaphragm, which is the prime cause of death because of asphyxia [128,129]. Currently, there are no known antidotes against acute guanidinium toxin poisoning. Curare blockers are not effective in alleviating the paralytic effects of these toxins, whereas anticholinesterase agents aggravate the symptoms. Potent stimulant benzedrine (DL amphetamine) has shown modest effects, shortening the recovery period [130]. Similar to numerous other biological toxins, the deadly effects of guanidinium toxins also conceal a major therapeutic potential, with their use in the management of drug-resistant pain currently under active investigation. The lack of TTX or STX effects on Na_V 1.5 channels, which is the main cardiac Na^+ channel, is especially attractive, ruling out adverse effects on myocardial function and cardiac rhythm [131]. Schwartz *et al.* demonstrated in rabbits that TTX produces local anesthesia that allowed corneal surgery [132], whereas others showed the use of TTX as a possible systemic analgesic for the treatment of long-term pain with neuropathic features [106]. Interestingly, local anesthetic bupivacaine or the neurotransmitter epinephrine not only reduced the systemic toxicity of TTX, but also intensified its potency for sensory blockade [131]. Nevertheless, in clinical trials, only 17 of 31 patients were positive for long-term pain relief [133]. The precise mechanisms of the antinociceptive effect of TTX and the basis of individual variability remain to be identified. Another area where TTX showed therapeutic promise is the ability to counter drug addiction. In rats, for instance, a decrease in demand for cocaine-induced stimuli was achieved by microinjections of TTX into specific brain regions [134]. Evidence from experiments on morphine-dependent mice and rats also suggested that low doses of TTX were valuable in the treatment of heroin dependence [134]. Finally, TTX is currently under active investigation as a treatment for cancer pain (Phase II/III; Wex Pharmaceuticals). Wex Pharmaceuticals and Esteve also have used TTX in Phase II trials for the treatment of opiate dependence [135]. Some purified STX analogs have also been applied as therapeutic candidates, and have shown considerable success in the treatment of chronic and acute anal fissures, by producing a flaccid paralysis of the anal muscle in a safe and effective way, thereby facilitating the healing process [136,137]. SXT has also shown promise in alleviating tension-type headache (TTH) [138] as well as in total knee arthroplasty (TKA), with no side effects or adverse reactions [139]. An *N*-1 hydroxylated STX analog (neosaxitoxin) was also used in the treatment of achalasia, a gastrointestinal motility disorder, resulting from failure of the lower esophageal sphincter, causing dysphagia or chest pain [140]. Sphincter relaxation by the local administration of small amounts of STX lasted for 2 days, leading to significant relief. The same mechanisms conceivably underlie the effects of STX as a local anesthetic [140] and as a long-acting pain blocker in the treatment of bladder pain syndrome, via infiltration of small doses

into the bladder submucosa, with no reported adverse effects over 90 days [97,141]. Overall, although promising therapeutic features have emerged for guanidinium toxin, further research is needed to understand their mechanisms of action and to expand their therapeutic utility.

α-Conotoxins targeting muscle nAChRs

α-Conotoxins are produced by marine organisms of the genus *Conus*, and present the richest source of potential drug leads known, with effects on diverse ion channels [142–145]. Produced as cocktails containing up to 200 conopeptides, they are used for prey capture via a variety of venom combinations within each of the 700 known *Conus* species. Most *Conus* species inhabit tropical and subtropical waters of the South China Sea, around Australia, and the Pacific Ocean [143]. Depending on their feeding habits, *Conus* species can be classified in three groups: (i) vermivorous (worm hunters); (ii) molluscivorous (mollusk hunters); and (iii) piscivorous (fish hunters) [146–148]. The first conotoxins were isolated in 1978 from *Conus geographus* venom [149], with >80 000 peptides identified to date. With the rapid advance of transcriptome and proteome sequencing, ~2000 mature conotoxins have recently been characterized in detail [150,151], with >70 transcriptomes from 30 *Conus* species published in the NCBI library [143,152]. Among this biodiversity, all *Conus* species known to date have at least one nAChR antagonist in their venom [153]. Most of these are disulfide-rich small peptides (12–19 amino acids), which act as α-neurotoxins [153]. α-Conotoxins comprise two loops: loop I comprises more similar and conserved amino acid residues (crucial for binding), and loop II is more variable and has a key role in selectivity binding [154].

Although α-conotoxins are classified based on their pattern of cysteine residues and disulfide connectivity (Cys1–Cys3 and Cys2–Cys4), the subclassification of α-conotoxins is based on the number of residues in the intercysteine loops. Hence, selective blockers of the muscle nAChR are dubbed α3/5-conotoxins, which means three residues in the first and five in the second loop, whereas subfamilies of blockers of neuronal nAChRs are entitled α4/7, α4/4, and α4/3 [145,153,155]. Among these, α-CTx GI from *Conus geografus* and α-CTx MI from *Conus magus* are the best-characterized α3/5-conotoxins, and can block specifically neuromuscular transmission both *in vitro* [156–158] and *in vivo* [159] (Table 4). Other muscle nAChR-blocking α-conotoxins with high selectivity have been isolated from *Conus striatus* (α-CTx SI, α-CTx SIA, and α-CTx SII) [160,161], *Conus consors* (α-CTx CnIA, and α-CTx CnIB) [162], and *Conus achatinus* (α-CTx Ac1.1a and α-CTx Ac1.1b) [163]. Interestingly, all these α-conotoxin variants have two binding sites on muscle nAChRs with different affinities, which vary depending on the prey species. Thus, α-CTx GI, α-CTx MI and α-CTx SIA are preferentially targeted to the α/δ interface of the muscle nAChR with a 10 000-fold higher affinity than for the α/γ interface [164–166]. By contrast, for the torpedo nAChR, all three peptides display higher affinity for α/γ compared with the α/δ interface [164]. Whereas α-CTx Ac1.1a and α-CTx Ac1.1b from *C. achatinus* block the mouse α/δ interface with a potency that is >50 000-fold higher than α/γ [163], α-CTx SI from *C. striatus* has a low affinity for both the α/γ and α/δ interfaces of the mouse muscle nAChR [167]. It has also less toxicity *in vivo* than either α-CTx GI or α-CTx MI and does not discriminate between the two binding sites on the torpedo nAChR [153,167,168]. Through mutagenesis studies, it was shown that the Arg residue at position 9 of α-CTx GI is crucial for the differential affinity of this toxin for the two binding sites of torpedo nAChR, also conferring a high affinity for the α/γ interface of mouse muscle nAChR [167,168]. α-CTx SI has a Pro instead of an Arg in that position, which could explain the differential pharmacology [167,168]. By contrast, a positively charged residue (either an Arg or Lys) at the C terminus of α-CTx GI, α-CTX SI, and α-CTX SIA appears to have an important role in enhancing the affinity for both binding sites on the torpedo nAChR [169].

Some α-conotoxins blocking the nAChR do not belong to the α3/5-conotoxin subfamily. For instance, α-CTx EI from *Conus ermineus*, which is a 'classical' α4/7-conotoxin by its structure, blocks some neuronal nAChRs as well as muscle nAChRs [170,171]. Interestingly, α-CTx EI blocks both the α/δ and α/γ interface with similar affinities, whereas α3/5 conotoxins preferentially block the mammalian α/δ interface and have a higher

TABLE 4

α3/5 and some more unusual muscle-specific α-conotoxins; sequence and receptor specificity[a,b]

Toxin	Species	Sequence	Receptor	Refs
α-CTx GI	*Conus geografus*	ECCNPACGRHYSC#	Muscle nAChR	[221]
α-CTx GIA	*C. geografus*	ECCNPACGRHYSCGK#	Muscle nAChR	[221]
α-CTx GII	*C. geografus*	ECCNPACGKHFSC#	Muscle nAChR	[221]
α-CTx MI	*Conus magnus*	GRCCHPACGKNYSC#	Muscle nAChR	[222]
α-CTx SI	*C. striatus*	ICCNPACGPKYSC#	Muscle nAChR	[223]
α-CTx SIA	*C. striatus*	YCCHPACGKNFDC#	Muscle nAChR	[160]
α-CTx SII	*C. striatus*	GCCCNPACGPNYGCGTSCŜ	Muscle nAChR	[161]
α-CTx CnIA	*Conus consors*	GRCCHPACGKYYSC#	Muscle nAChR, α7 nAChR	[162]
α-CTx CnIB	*C. consors*	CCHPACGKYYSC#	Muscle nAChR	[162]
α-CTx Ac1.1a	*Conus achatinus*	NGRCCHPACGKHFNC#	Muscle nAChR	[163]
α-CTx Ac1.1b	*C. achatinus*	NGRCCHPACGKHFSC#	Muscle nAChR	[163]
α-CTx EI	*Conus ermineus*	RDOCCYHPTCNMSNPQIC#	Muscle nAChR, α3β4 nAChR, α4β2 nAChR (potentiation)	[97,170]
α-CTx SrIA	*Conus spurius*	RTCCSROTCRMγYPγLCG#	Muscle nAChR, α4β2 nAChR (potentiation)	[170]
α-CTx SrIB	*C. spurius*	RTCCSROTCRMEYPγLCG#	Muscle nAChR, α4β2 nAChR (potentiation)	[170]
α-CTx PIB	*Conus purpurascens*	ZSOGCCWNPACVKNRC#	Adult/fetal muscle nAChR	[172]

[a] The cysteine residues that form the disulfide bridges are in bold.
[b] #, amidated C terminus; ^, free carboxyl C terminus; γ, γ-carboxyglutamine; O, 4-trans-hydroproline; Z, pyroglutamate.

affinity for the α/δ interface of the torpedo nAChR, in contrast to the α/γ-interface-preferring α3/5- conotoxins [171]. Two other α4/7-conotoxins, SrIA and SrIB, have been discovered from *Conus spurius*, which blocks the muscle nAChR with greater potency than nAChRs [170]. Surprisingly, α-CTx GI, α-CTX SI, and α-CTX SIA have potentiating effects on the α4β2 nAChR [170]. In 2007, an unusual muscle-specific α-conotoxin, α-CTx PIB, was purified from the venom of *Conus purpurascens* [172], and is arranged by 4/4 intercysteine loop spacing. It is capable of blocking both adult and fetal mouse muscle nAChRs expressed in oocytes, although with insignificant effects on neuronal subtypes. Overall, the diversity of α-conotoxins with a broad range of activity makes them attractive drug leads, with some undergoing preclinical evaluation for the treatment of chronic and neuropathic pain [173,174]. From 1998 to 2017, 811 patents were registered on official sites concerning the clinical development of drugs from conotoxins, with 243 authorized [143,175]. The treatment of multiple diseases, including pain, Alzheimer's disease, Parkinson's disease, cardiac infarction, hypertension, and various neurological diseases [175–177], by conotoxins are now undergoing in-depth preclinical research and validation in animal models.

Irreversible activators of Na^+ channels at NMJs: batrachotoxin alkaloids

Among the activators of Na_V channels, batrachotoxin (BTX) has been the best characterized. It was first purified from the poisonous *Phyllobates* frog, which inhabits temperate rain forests [178]. Subsequently, BTX was also identified in several species of New Guinean birds (genera *Pitohui* and *Ifrita*) [179–181] and beetles (genus *Choresine*) [182], albeit in small quantities (e.g., ~1.8 μg per adult beetle). In dendrobatid frogs, three major BTX alkaloids have been identified: highly potent BTX and homobatrachotoxin, and their less toxic precursor, BTX A. In captivity, *Phyllobates* lose their skin toxicity, suggesting that the poisonous substance accumulates in the animals because of a specific diet, potentially bound to certain insects that contribute to the diet of the frogs. BTX is the strongest known activator of Na^+ channels and is an extremely potent neurotoxin (LD_{50} = 1–2 mg/kg subcutaneous in mice). Studies of its *in vitro* activity reported BTX EC_{50} ranging from 0.1 to 100 nM concentration [183]. Chemically, BTX is an alkaloid with a dimethylpyrrole carboxylate moiety and oxazapine ring [184,185], containing a rigid steroidal core structure that is formed by a tertiary amine with 538 Da, pK_a ~7.5 (Fig. 6a) [186].

In stark contrast to the above-discussed guanidinium toxins, which act on Na^+ channels as potent blockers, BTX with its steroid skeleton acts as a potent and irreversible activator of the Na_V channel, through stable binding with the site 2 of the third domain of the α-subunit (Fig. 6d,e). BTX-sensitive Na_V channels are widely expressed in neurons of the central and peripheral nervous systems, as well as in striated and cardiac muscles, where they regulate membrane excitability. Studies using photo-labeled BTX identified the localization of the binding residues within the

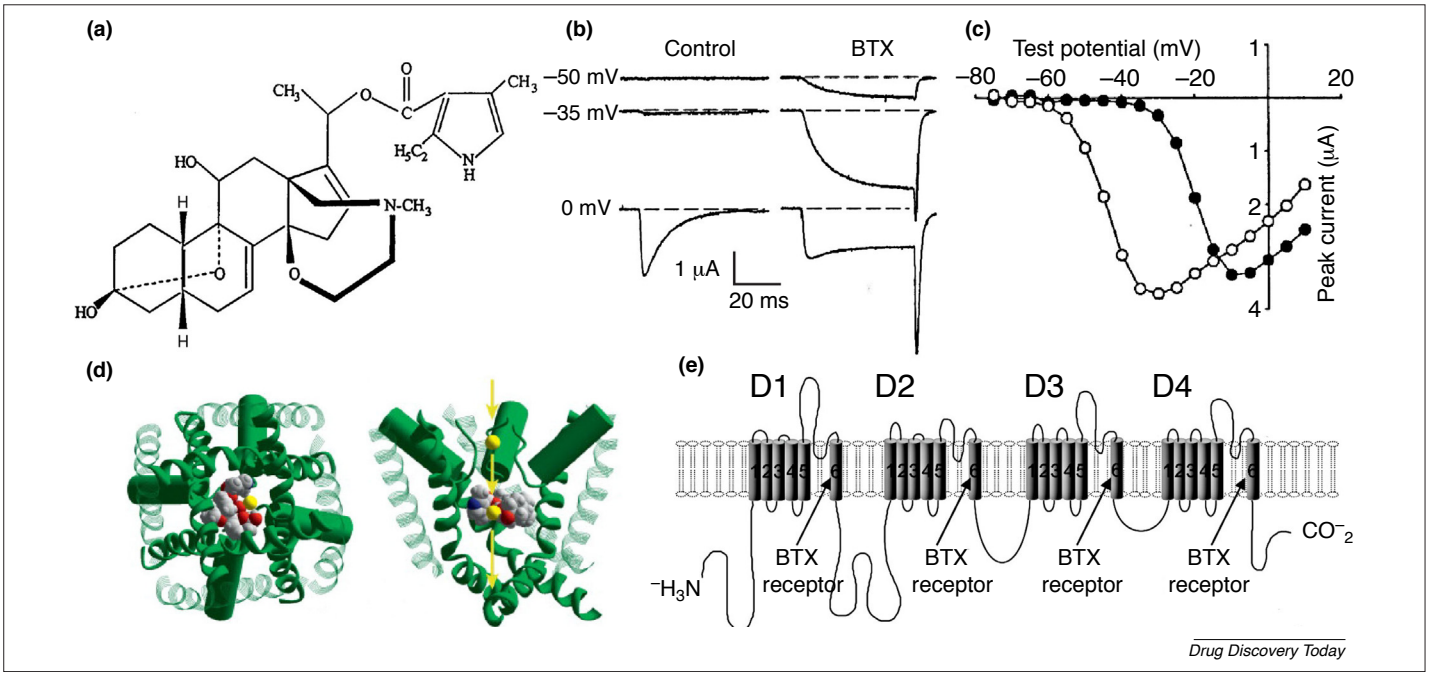


FIGURE 6

Selective and potent enhancement of Na^+ currents with batrachotoxin (BTX). **(a)** Chemical structure of BTX. **(b)** BTX modifies the properties of cloned $Na_V1.3$ channels expressed in oocytes: typical whole-cell Na^+ currents elicited in control conditions (left) and after 35 ms pulses to 0 mV in the presence of BTX (right traces) at specified holding potentials. The example traces show currents evoked by 70 ms depolarization to −50, −35, and 0 mV. In all cases, the holding potential was −90 mV and the concentration of BTX was 10 μM. **(c)** Complete current–voltage relationships under control conditions and BTX treatment (black and white circles, respectively). The data points show peak amplitude of currents elicited by depolarization to test potential from −80 to +10 mV, plotted as a function of test potential. **(d)** Cytoplasmic and side view of the Na^+ channel with BTX, showing that the bulky toxin binds in the central cavity, but does not block because the permeant ion can pass through an interface formed by three oxygen atoms of the toxin and hydrophilic residues of the channel. **(e)** Transmembrane topology of the voltage-gated sodium channel α-subunit. The cylinders embedded in the lipid bilayer represent the transmembrane α-helical segments (S1–S6) of four domains of the channel. Arrows indicate the BTX receptor at the inner cavity. Adapted, with permission, from [201] (c) and [202] (d); modified from [186] (e).

inner helixes of DI S6 and DIV S6 of the $Na_V1.4$ and 1.5 subunits [187]. Such binding to the inner pore causes a multitude of electrostatic changes, including a shift in the voltage-dependence of activation plus attenuation of both fast and slow inactivation, with a strong shift towards hyperpolarizing potentials [188]. The action of BTX is mediated by binding channels in the open state; keeping the pore persistently open, even at negative potentials, prevents channel inactivation (Fig. 6b,c). Such effects are associated with the loss of selectivity of Na_V channels, facilitating the permeability of larger ions through the channel pore. Poisonous frogs are somewhat resistant to BTX and its analogs, because of amino acid replacements in five different positions in the inner pore of Nav1.4 [189]. Recently, it was demonstrated that a single amino acid substitution (asparagine→threonine) via targeted mutagenesis caused complete resistance of muscle Na^+ channels to the most toxic BTX of *Phyllobates terribilis* [186]. In addition, BTX A, a synthetic derivative of native BTX, with minor chemical differences (namely lacking the C-20 pyrrole ester), has lower toxicity (LD_{50} = 1000 mg/kg subcutaneous in mice) [190]. These findings are in general agreement with the results of earlier studies of the effects of semisynthetic BTX on Na_V channels, demonstrating the importance of the positioning and location of the amine and ester groups, in addition to oxygen functional groups at C3, C9, and C11 positions for toxicity [191]. Surprisingly, some enantiomers of BTX, which demonstrate nearly identical potency to wild-type variants, act as irreversible blockers of Na_V channels.

From a translational standpoint, the high selectivity and unique ability to facilitate Na_V currents render wild-type BTX a useful research tool for probing the molecular identity and stoichiometry of Na^+ channels, as well as the characterization of their distribution and binding sites [187]. BTX has also shown an ability for allosteric modulation of Na^+ channels to other toxins [192]. For instance, the synergistic effect of BTX for channel binding has been reported for scorpion α-toxins, facilitating its binding to receptor site 3. Likewise, binding of pyrethroid insecticides to receptor site 7 and/or of brevetoxin binding to receptor site 5 have been enhanced by BTX [193]. Similar effects of BTX have also been described for several synthetic drugs, including well-known anticonvulsants, antiarrhythmic, and antidepressants therapies, as well as analgesics and anesthetics [187], implying its major research and translational potential. Enhancement of Na^+ channel functions by BTX has also potential applications for countering loss-of-function mutations of Na_V, which include a range of genetic disease causing inherited forms of periodic paralysis, cardiac arrhythmia, epilepsy, and chronic pain. BTX could also be used for countering neurological signs of Dravet syndrome (and possibly also for some other generalized epilepsy with febrile seizures), where loss-of-function mutations in $Na_V1.1$ channels selectively dampen the excitability of GABAergic inhibitory neurons, thereby driving the hyperexcitability of neural circuits, causing epilepsy [194]. In hypokalaemic periodic paralysis, where mutations of the gating charges in the voltage sensor of $Na_V1.4$ channels cause an ionic leak, resulting in excess of intracellular Na^+ and depolarization followed by conduction blockade and episodic paralysis, modified BTX proved useful for restoring Na_V channel normal functionality. The local anesthetic binding site, where all clinically applied Na^+ channel blockers are thought to bind, is also known to partially overlap with the BTX interaction site [135]. Thus, BTX was used for establishing structure–activity relationships among newly synthesized Na^+ channel blockers (i.e., the [3 H]-BTX binding assay), in which [3 H]-batrachotoxinin A 20-a-benzoate is applied as a radioligand to characterize the pharmacodynamic properties of blockers. Finally, with respect to its unique properties and ready transformation from agonist to the antagonist of Na_V channels, because of minor changes in its chemical structure, BTX has become increasingly attractive as a lead compound for the design of Na^+ channel modulators, also acting at the NMJ and striatal muscles [186].

Concluding remarks and future directions

Since ancient times, remedies and poisons based on natural venoms and toxins have been widely used, but only recently have their mechanisms of action become understood, uncovering their enormous therapeutic potential. Among these, toxins targeting peripheral synapses at the interface of motor nerve endings and muscles hold a unique position, because of the ready accessibility of NMJs beyond the blood–brain barrier. The NMJ also represents the primary entry site for a range of toxins and pathogens into motor neurons and, hence, presents a gateway for therapeutic delivery to the central nervous system via retrograde transport. With the arrival of advanced molecular biology and genetics tools, an in-depth understanding has been gained into the exquisite mechanisms of synaptic transmission and ion channels operating at this crucial functional juncture. Rapidly mounting knowledge over recent years has not only facilitated progress in the understanding of fundamental mechanisms operating at the NMJ, but also revealed a roadmap for targeting and manipulation of specific processes therein, using rational drug design approaches, including pharmacological leads based on neurotoxins and their derivatives.

In the context of NMJ studies, areas such as dystonia, periodic or persistent muscular weakness and hypersensitization because of abnormal cholinergic tonus have been of particular interest. As discussed, curare and curaremimetics with excellent selectivity for the nAChR and advantageous pharmacokinetic and pharmacodynamic properties present considerable interest and potential as muscle relaxants with antinociceptive potential, offering better controlled and safer anesthesia, independent from relaxant effects. By contrast, fasciculins, with selective and potent inhibitory effects on AChE, offer opportunities for boosting the cholinergic tone at NMJs and beyond, with the potential also for enhancement of ACh activity in central synapses. At NMJs, these effects show promise in countering flaccid paralysis and muscle weakness, whereas, in the central nervous system, fasciculins show potential as cognitive enhancers, through augmentation of cholinergic activity, known to regulate cognition and memory. Finally, guanidinium-containing toxins blocking Na_V channels at the periphery have been investigated in the context of chronic pain management, particularly in treating painkiller-resistant forms. Unlike most conventional pain-alleviating medications with numerous adverse effects, especially prominent during extended usage, the utility of guanidinium toxins show no evidence of acclimation or addiction, exhibiting dual effects on nerves and muscle tissues at the administration site. Currently, basic and translational research is underway to develop toxin-based biopharmaceutics with greater specificity, to meet pressing medical needs for low-cost and effective drugs with minimal adverse effects.

Overall, the emerging data from preclinical and clinical studies suggest that natural toxins targeting NMJs conceal major potential not only as valuable research tools, but also as leads for drug discovery. Given that AChR, AChE, and Na_V malfunction underlies a range of diseases affecting motor neurons and NMJs, as well as epilepsy, neuropathic pain, and long QT syndrome, there is a pressing need and interest in drug leads that interfere in one way or another with mechanisms underlying these conditions. In this context, research into the effects of venoms at NMJs has proved to be highly instructive. As illustrated throughout this review, the boundaries between poison and medicine are fluid, as envisaged by Paracelsus centuries ago, offering enormous opportunities for modern drug discovery.

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