

Review

Alpha-conotoxins as pharmacological probes of nicotinic acetylcholine receptors

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Cysteine-rich peptides from the venom of cone snails (*Conus*) target a wide variety of different ion channels. One family of conopeptides, the α -conotoxins, specifically target different isoforms of nicotinic acetylcholine receptors (nAChRs) found both in the neuromuscular junction and central nervous system. This family is further divided into subfamilies based on the number of amino acids between cysteine residues. The exquisite subtype selectivity of certain α -conotoxins has been key to the characterization of native nAChR isoforms involved in modulation of neurotransmitter release, the pathophysiology of Parkinson's disease and nociception. Structure/function characterization of α -conotoxins has led to the development of analogs with improved potency and/or subtype selectivity. Cyclization of the backbone structure and addition of lipophilic moieties has led to improved stability and bioavailability of α -conotoxins, thus paving the way for orally available therapeutics. The recent advances in phylogeny, exogenomics and molecular modeling promises the discovery of an even greater number of α -conotoxins and analogs with improved selectivity for specific subtypes of nAChRs.

Keywords: *Conus*; nicotine; alpha-conotoxin; muscle nicotinic acetylcholine receptor; neuronal nicotinic acetylcholine receptor; *Torpedo*; AChBP

Acta Pharmacologica Sinica (2009) 30: 771–783; doi: 10.1038/aps.2009.47; published online 18 May 2009

Introduction

Marine organisms belonging to the genus *Conus* are a rich source of pharmacological agents that act on diverse ion channels^[1–5]. These agents, known as conopeptides, have evolved as selective tools for capture of prey or deterrence of predators. The ~500 known different *Conus* species each contain greater than 100 different components in their venom, leading to an estimated >50 000 different pharmacologically active conopeptides.

The best characterized biologically-active venom components are conotoxins, small, disulfide-rich peptides, most of which are specifically targeted to different voltage- or ligand-gated ion channels^[3,6]. One rationale for the vast diversity of different conotoxins found in *Conus* venom is that this is an evolutionary consequence of the remarkable diversity of molecular isoforms of ion channels. Each *Conus* species uses the equivalent of several drug combinations to rapidly capture its prey. This review focuses on one family of com-

pounds in *Conus* venom referred to as α -conotoxins, which are nicotinic acetylcholine receptor (nAChR) antagonists.

All *Conus* species characterized thus far have at least one nAChR antagonist in their venom^[7]. The largest family of such antagonists are the α -conotoxins, small, disulfide rich peptides generally 12–19 amino acids in length^[8,9]. The α -conotoxins are classified based on their cysteine pattern CC-C-C, with a disulfide connectivity of Cys1-Cys3 and Cys2-Cys4. There is a further sub-classification of α -conotoxins based on the number of residues in their inter-cysteine loops (see Figure 1). The α 3/5-conotoxins (3 residues in the first and 5 in the second loop) are selective blockers of the muscle nAChR, whereas the α 4/7, α 4/4, and α 4/3 subfamilies are generally blockers of neuronal nAChRs^[3,7,8]. Although this review focuses primarily on neuronally targeted α -conotoxins, the currently known muscle nAChR targeted α -conotoxins are briefly discussed below (also see Table 1).

Muscle nAChR-targeted α -conotoxins

The first α -conotoxins to be purified from *Conus* venom

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Received 2009-02-16 Accepted 2009-03-24

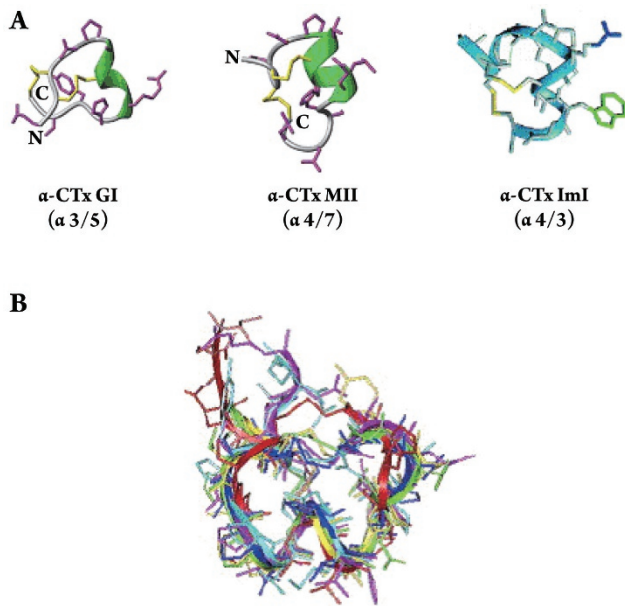


Figure 1. Backbone structure of subfamilies of α -conotoxins. (A) Backbone structure of an α 3/5 conotoxin (α -CTx GI), an α 4/7 conotoxin (α -CTx MII) and an α 4/3 conotoxin (α -CTx ImI). For α -CTx GI and α -CTx MII: green represents the α -helix; yellow, disulfide bonds; purple, side chain of toxin residues. For α -CTx ImI: light blue represents backbone structure; yellow, disulfide bonds; dark blue, side chain of Arg7; green, side chain of Trp10; gray, other “non-essential” toxin residues. (B) Superimposed ribbon diagrams of six α 4/7-conotoxins: α -conotoxins PIA (violet), MII (blue), GIC (green), PnIA (yellow), GID (red), and EI (cyan). Although members of each subfamily have similar backbone structures (as shown in B), members across different subfamilies have different structures (shown in A). In A, α -CTx GI and α -CTx MII are from [5], α -CTx ImI is from [136]. Superimposed structures in B is from [137]. All images reproduced by permission from Elsevier, Inc through Rights Link Copyright Clearance Center.

Table 1. Sequence and receptor specificity of α 3/5 and other more unusual muscle-specific α -conotoxins. #, amidated C-terminus; ^, free carboxyl C-terminus; γ , γ -carboxyglutamate; O, 4-*trans*-hydroxyproline; Z, pyroglutamate. The cysteine residues that form the disulfide bridges are in bold.

Toxin	Sequence	Receptor	Reference
α -CTx GI	ECCNPACGRHYSC#	Muscle	[10]
α -CTx GIA	ECCNPACGRHYSCGK#	Muscle	[10]
α -CTx GII	ECCNPACGKHFSC#	Muscle	[10]
α -CTx MI	GRCCHPACGKNYSC#	Muscle	[17]
α -CTx SI	ICCNPACGPKYSC#	Muscle	[18]
α -CTx SIA	YCCHPACGKNFDC#	Muscle	[19]
α -CTx SII	GCCNPACGPNYCGTSCS^	Muscle	[20]
α -CTx CnIA	GRCCHPACGKYSC#	Muscle, α 7	[21]
α -CTx CnIB	CCHPACGKYSC#	Muscle	[21]
α -CTx Ac1.1a	NGRCCHPACGKHFNC#	Muscle	[22]
α -CTx Ac1.1b	NGRCCHPACGKHFSC#	Muscle	[22]
α -CTx EI	RDCCYHPTCNMSNPQIC#	Muscle, α 3 β 4, α 4 β 2 (potentiation)	[34, 36]
α -CTx SrIA	RTCCSROTCRM γ YP γ LCG#	Muscle, α 4 β 2 (potentiation)	[36]
α -CTx SrIB	RTCCSROTCRM γ YP γ LCG#	Muscle, α 4 β 2 (potentiation)	[36]
α -CTx PIB	ZSOGCCWNPACVKNRC#	Adult/fetal muscle	[37]

were α -conotoxins GI, GIA, and GII from the fish-hunting cone snail *Conus geographus*^[10]. The best characterized is α -CTx GI, which blocks neuromuscular transmission both *in vitro*^[11–13] and *in vivo*^[14], but does not block any of the neuronal nAChR subtypes^[15, 16]. α -CTx MI, from *Conus magus*, was subsequently isolated and characterized as another blocker of the ACh responses at the neuromuscular junction^[17]. Subsequently, a series of muscle nAChR blocking α -conotoxins were isolated from *Conus striatus*, α -CTx SI, α -CTx SIA, and α -CTx SII^[18–20], *Conus consors*, α -CTx CnIA,

and α -CTx CnIB^[21] and *Conus achatinus*, α -CTx Ac1.1a and α -CTx Ac1.1b^[22].

Binding data on mouse muscle receptors demonstrate that α -CTx GI, α -CTx MI, and α -CTx SIA are preferentially targeted to the α / δ interface of the muscle nAChR (with an affinity >10 000 fold higher than for the α / γ interface^[23–25]). In contrast, for the *Torpedo* nAChR, all three peptides display much higher affinity for the α / γ vs the α / δ interface^[24]. Two newly discovered toxins from *Conus achatinus*, α -CTx Ac1.1a, and α -CTx Ac1.1b, block the mouse α / δ interface with a

potency that is >50 000 fold higher than α/γ ^[22]. In contrast, α -CTx SI has low affinity for both the α/γ and α/δ interfaces of mouse muscle nAChR, and does not discriminate between the two sites as well as either α -CTx GI or α -CTx MI^[24]. α -CTx SI has less toxicity *in vivo* than either α -CTx GI or α -CTx MI^[18]. In addition, α -CTx SI does not discriminate between the two binding sites on the *Torpedo* nAChR^[26, 27]. Structure-activity studies have indicated that Arg at position 9 of α -CTx GI is responsible for the differential affinity of this toxin for the two binding sites of the *Torpedo* nAChR^[28]; this residue also confers high affinity for the α/γ interface of mouse muscle nAChR^[27]. Instead of Arg, α -CTx SI has a Pro in the homologous position, which may account for its differential pharmacology^[27, 28]. Comparison of solution structures of α -CTx SI with α -CTx GI suggests that it is the loss of the basic charge, rather than differences in backbone structure, that underlies the pharmacological differences between the two toxins^[29]. The residues on the mouse δ subunit that confer high binding affinity to α -CTx MI have been determined and include Ser36, Tyr113, and Ile178^[30]. The α -CTx MI residues that interact with the receptor binding pocket have also been determined^[30-32]. In addition, a recent study has indicated the importance of a positively charged residue (either an Arg or Lys) at the C-terminus of α -CTx GI, α -CTx SI, and α -CTx SIA in enhancing affinity for both binding sites on the *Torpedo* nAChR^[33].

Although all of the “classical” muscle nAChR blocking α -conotoxins belong to the $\alpha 3/5$ -conotoxin subfamily, several muscle nAChR blocking α -conotoxins belong to other branches of the α -conotoxin family (Figure 1). α -CTx EI, isolated from the fish-hunting Atlantic species *Conus ermineus*^[34], is an $\alpha 4/7$ -conotoxin, a subfamily that is usually associated with blocking neuronal nAChRs. The solution structure of α -CTx EI is similar to other $\alpha 4/7$ conotoxins (Figure 1)^[35]. The backbone structure of $\alpha 4/7$ conopeptides is illustrated in Figure 1. Although α -CTx EI does block some neuronal nAChRs^[36], unlike other $\alpha 4/7$ conotoxins it's also a potent blocker of the muscle nAChR^[34, 36]. Unlike the $\alpha 3/5$ conotoxins that preferentially block the mammalian α/δ interface (see above), α -CTx EI blocks both the α/δ and α/γ interface with similar affinity^[34]. Moreover, α -CTx EI has higher affinity for the α/δ interface of the *Torpedo* nAChR, in contrast to the α/γ -interface preferring $\alpha 3/5$ -conotoxins^[34]. Recently, two other $\alpha 4/7$ -conotoxins that block the muscle nAChR, and to a lesser extent neuronal nAChRs, have been discovered, α -conotoxins SrIA and SrIB from *Conus spurius*^[36]. Similar to α -CTx EI, α -CTx SrIA and α -CTx SrIB contain post-translational modifications in their native sequences. Unlike other $\alpha 4/7$ conotoxins, however,

α -CTx SrIA, and SrIB are reported to have nAChR potentiating activity on the $\alpha 4\beta 2$ subtype^[36].

Another unusual muscle-specific α -conotoxin is α -CTx PIB, which has an uncommon 4/4 intercysteine loop spacing^[37]. It was purified from the venom of *Conus purpurascens* and found to block both adult and fetal mouse muscle nAChRs expressed in oocytes with nanomolar potency, with little effect on any neuronal subtypes^[37].

Neuronal nAChR-targeted α -conotoxins

The α -conotoxins targeting neuronal nAChRs are numerous and have even more exquisite subtype selectivity, probably due to the large diversity of isoforms in this subfamily of receptors. The members of this family of toxins that have been discovered thus far are listed below, in no particular order (also see Tables 2 and 3).

α -Conotoxins ImI and ImII The first α -conotoxin targeting neuronal nAChRs was α -CTx ImI. It was also the first α -conotoxin isolated from the venom of a worm hunting species, *Conus imperialis*^[38]. This α -conotoxin has the unusual inter-cysteine loop spacing 4/3 (Figure 1A). This peptide was found to be inactive when injected interaperitoneally, whereas intracerebral injection caused seizures and death^[38]. In addition, this toxin blocks nicotinic responses of B cells on frog sympathetic ganglia but not mammalian neuromuscular nAChRs^[38, 39]. Subsequent pharmacological characterization on heterologously expressed nAChRs showed an IC₅₀ of 220 nmol/L on homomeric $\alpha 7$ nAChRs and 1.8 μ mol/L on homomeric $\alpha 9$ nAChRs^[16]. Ellison *et al* (2004) reported an IC₅₀ of 595 nmol/L on homomeric $\alpha 7$ nAChR, but they also showed potent inhibition of heteromeric $\alpha 3\beta 2$ nAChR (IC₅₀ 41 nmol/L). The seizure inducing effects of this toxin are presumably due to its block of α -bungarotoxin-sensitive $\alpha 7$ nAChRs on hippocampal neurons^[40]. Structure-activity studies have suggested a role for residues in the first loop of the toxin, Asp5, Pro6, and Arg7, as well as Trp10 in the second loop, in interacting with the $\alpha 7$ subunit^[41]. Homology modeling using *Aplysia* AChBP, a structural homolog of the N-terminal binding region of nAChRs, confirms a key role for Arg7 and Trp10 in interaction with residues at the ligand binding site^[42, 43]. Co-crystallization of α -CTx ImI with *Aplysia* AChBP reveals a more open C-loop to accommodate the α -conotoxin^[44]. The $\alpha 7$ subunit residues that interact with the toxin have also been determined^[45, 46].

In recent years, attempts have been made to improve the biological stability of α -conotoxins by protecting the disulfide bonds against reduction or scrambling due to exposure to intra- or extracellular environments, such as blood. One

Table 2. Sequence and receptor specificity of neuronal-specific α -conotoxins. # denotes an amidated C-terminus; ^, free carboxyl C-terminus; γ , γ -carboxyglutamate; O, 4-*trans*-hydroxyproline; Y(S), sulfotyrosine. The cysteine residues that form the disulfide bridges are in bold.

Toxin	Sequence	Receptor	Reference
α -CTx ImI	GCCSDPRCAWRC#	$\alpha 3\beta 2 > \alpha 7 > \alpha 9$	[16, 38, 49, 50]
α -CTx ImII	ACCSDRRCRWRC#	$\alpha 7$	[49, 50]
α -CTx MII	GCCSNPVCHLEHSNLC#	$\alpha 3\beta 2 \approx \alpha 6\beta 2^* > \alpha 6\beta 4$	[51–53]
α -CTx PIA	RDPCCSNPVCTVHNPQIC#	$\alpha 6\beta 2^* > \alpha 6\beta 4 \approx \alpha 3\beta 2$	[87]
α -CTx OmIA	GCCSHPACNVNPNPHICG#	$\alpha 3\beta 2 > \alpha 7 > \alpha 6\beta 2^*$	[88, 89]
α -CTx GIC	GCCSHPACAGNNQHIC#	$\alpha 3\beta 2 \approx \alpha 6\beta 2^* > \alpha 7$	[90, 91]
α -CTx GID	IRDγCCSNPACRVNNOHVC[^]	$\alpha 7 \approx \alpha 3\beta 2 > \alpha 4\beta 2$	[92, 93]
α -CTx EpI	GCCSDPRCNMNNPDY(S)C#	$\alpha 3^*, \alpha 7$	[94, 95]
α -CTx AnIA	CCSHPACAANNQDY(S)C#	$\alpha 3\beta 2, \alpha 7$	[96]
α -CTx AnIB	GGCCSHPACAANNQDY(S)C#	$\alpha 3\beta 2, \alpha 7$	[96]
α -CTx AuIA	GCCSYPPCFATNSDYC#	$\alpha 3\beta 4$	[72]
α -CTx AuIB	GCCSYPPCFATNPDC#	$\alpha 3\beta 4$	[72]
α -CTx AuIC	GCCSYPPCFATNSGYC#	$\alpha 3\beta 4$	[72]
α -CTx PnIA	GCCSLPPCAANNPDY(S)C#	$\alpha 3\beta 2 > \alpha 7$	[98–101]
α -CTx PnIB	GCCSLPPCALSNPDY(S)C#	$\alpha 7 > \alpha 3\beta 2$	[98–101]
α -CTx BuIA	GCCSTPPCAVLYC#	$k_{\text{off}} \beta 2 > k_{\text{off}} \beta 4$	[106]
α -CTx PeIA	GCCSHPACSVNHPELC#	$\alpha 9\alpha 10 > \alpha 3\beta 2 > \alpha 6\beta 2^* > \alpha 3\beta 4 > \alpha 7$	[112]
α -CTx Vc1.1	GCCSDPRCNVDHPEIC#	$\alpha 9\alpha 10 > \alpha 6\beta 2^* > \alpha 6\beta 4 > \alpha 3\beta 4 \approx \alpha 3\beta 2 > \alpha 7$	[114–117]
α -CTx RgIA	GCCSDPRCRYRCR[^]	$\alpha 9\alpha 10 > \alpha 7$	[113, 116]
α -CTx TxIA	GCCSRPPCIANNPDLC#	AChBP $\approx \alpha 3\beta 2 > \alpha 7$	[124]
α -CTx Lp1.1	GCCARAACAGIHQELC#	$\alpha 3\beta 2, \alpha 6\beta 2$	[125, 126]
α -CTx ArIA	IRDECCSNPACRVNNOHVCRRR[^]	$\alpha 7 \approx \alpha 3\beta 2$	[128]
α -CTx ArIB	DECCSNPACRVNPNPHVCRRR[^]	$\alpha 7 \approx \alpha 6\beta 2^* > \alpha 3\beta 2$	[128]

Table 3. Rank order of activity of α -conotoxins on select nAChR subtypes.

nAChR subtype	α -Conotoxin rank order	Reference
$\alpha 3\beta 2$	MII \approx GIC \approx GID \approx AnIB $>$ BuIA \approx OmIA \approx PnIA \approx ArIA $>$ PeIA $>$ ImI \approx ArIB \approx PIA	[50, 87–89, 92, 100, 106, 112, 128]
$\alpha 6\beta 2^*$	MII[E11A] \approx BuIA \approx MII $>$ PIA \approx MII[S4A; E11A; L15A] \approx GIC \approx MII[H9A; L15A] $>$ ArIB $>>$ Vc1.1 \approx OmIA	[76, 87, 88, 106, 116, 128]
$\alpha 3\beta 4$	BuIA $>$ AuIB \approx PIA \approx PeIA	[72, 87, 106, 112]
$\alpha 6\beta 4$	BuIA $>$ MII \approx PIA $>$ Vc1.1	[87, 106, 116]
$\alpha 4\beta 2$	GID	[92]
$\alpha 7$	ArIB[V11L,V16D] \approx ArIB \approx GID $>$ PnIA[A10L] $>$ OmIA $>$ PnIB $>$ PnIA \approx ImI \approx ImII	[50, 89, 92, 100, 128, 129]
$\alpha 9\alpha 10$	RgIA \approx PeIA $>$ Vc1.1	[112, 116]

of these studies utilized selenocysteines in place of cysteines to yield several selenoconotoxin analogs of α -CTx ImI^[47]. Although similar to wildtype α -CTx ImI in both structure and activity against $\alpha 7$ nAChRs, the selenoconotoxin analogs

were more stable than wildtype α -CTx ImI under a variety of chemical and biological reducing conditions^[47]. A second method of improving conformational stability is to replace the cystine bridges with non-reducible dicarba linkages. The dicarba analog of α -CTx ImI had a similar structure to wildtype α -CTx ImI, with a slight difference in the geometry of disulfide *vs* dicarba bridges. The activity of the analog against $\alpha 7$ nAChR was similar to wildtype α -CTx ImI^[48].

Another $\alpha 4/3$ conotoxin from the venom of *Conus imperialis*, α -CTx ImII, was identified using a PCR-based discovery strategy^[49]. Although highly homologous to α -CTx ImI in sequence (9 out of 12 residues are shared), α -CTx ImII, unlike α -CTx ImI, does not compete with α -bungarotoxin for binding to heterologously expressed $\alpha 7$ nAChRs, suggesting a distinct and perhaps novel binding site on the $\alpha 7$ nAChR for α -CTx ImII^[50]. The difference in binding of the two toxins is due to the presence of a Pro at position 6 of α -CTx ImI, which has been shown to be important for interaction of this toxin with $\alpha 7$ nAChRs^[41]. α -CTx ImII has an Arg at this position; mutation of this Arg to a Pro creates an analog that competes with α -bungarotoxin binding^[49].

α -Conotoxins MII, PIA, OmIA A second α -conotoxin isolated from *Conus magus* was α -CTx MII. It belongs to

the $\alpha 4/7$ subfamily (Figure 1). α -CTx MII blocks the $\alpha 3\beta 2$ nAChR subtype with a potency 2–4 orders of magnitude higher than most other nicotinic receptors, including the muscle subtype^[51]. However, binding studies in knock-out animals^[52] as well as functional studies^[53], have shown that α -CTx MII also potently acts at $\alpha 6$ -containing nAChRs. Subsequently, a series of α -CTx MII analogs were made that selectively target the chimeric $\alpha 6/\alpha 3\beta 2\beta 3$ ^[54] vs $\alpha 3\beta 2$ nAChR^[53]. These toxin analogs are the most selective $\alpha 6^*$ nAChR antagonists reported to date. The wildtype α -CTx MII, as well as a number of $\alpha 6^*$ (the asterisk indicates the presence of additional subunits) selective α -CTx MII analogs, have been used to characterize nAChR subtypes that modulate dopamine release in rat^[55–61], mice^[62–65], and monkey striatum^[66,67]. These studies indicate a role for $\alpha 6\beta 2^*$ and $\alpha 6\alpha 4\beta 2^*$ nAChRs in the modulation of dopamine release in the striatum and the nucleus accumbens. The contribution of both the $\alpha 4$ and the $\beta 3$ subunits to α -CTx MII binding sites in dopaminergic neurons has been confirmed in studies using knock-out mice^[64,68,69]. Some studies also suggest a selective downregulation of $\alpha 6^*$ nAChRs upon chronic nicotine exposure^[70,71]. α -CTx MII has also been used to characterize distinct nAChRs that modulate [³H]NE release in rat^[72] vs mouse hippocampus^[73]. Table 4 summarizes some of the nAChR-mediated physiological functions characterized using the α -conotoxins.

Structure-activity studies have identified the amino acid residues on the $\alpha 3$ and the $\beta 2$ nAChR subunits that interact with α -CTx MII. These include Lys185 and Ile188 on the $\alpha 3$ subunit and Thr59, Val109, Phe117 and Leu119 on the $\beta 2$ subunit^[74,75]. In addition, α -CTx MII[S4A; E11A; L15A] has been used to identify the nAChR subunit amino acid residues that interact with, and confer selectivity, for the $\alpha 6$ vs the $\alpha 3$ subunit^[76]. Notably, these nAChR subunit residues are distinct from those that interact with wildtype α -CTx MII^[74].

Radiolabeling of α -CTx MII has allowed direct measurement of the binding sites for this α -conotoxin within the

brain^[77]. Fluorescently-labeled analogs of α -CTx MII have also recently been synthesized^[78]. Using the radiolabeled α -CTx MII, Quik and co-workers have found a selective downregulation of α -CTx MII binding sites within rodent and monkey striatum after nigrostriatal damage^[79–82] as well as in humans with Parkinson's^[82]. Direct measurement of α -CTx MII sites also shows preferential recovery of these sites in monkeys allowed to recover from nigrostriatal damage^[66]. Binding studies using an analog of α -CTx MII, α -CTx MII[E11A], have shown a selective loss of a specific subtype of nAChR ($\alpha 6\alpha 4\beta 2^*$) in rodent and monkey models of Parkinson's disease. These studies were replicated in post-mortem tissue from humans with Parkinson's^[83]. Thus, this α -CTx MII analog has a nAChR subtype selectivity that has potential in the early diagnosis of Parkinson's disease.

A number of attempts have been made to enhance the stability of α -CTx MII against proteolysis as well to improve its lipophilicity, with the aim of increasing the oral bioavailability of this toxin. Backbone cyclization by use of linker placement and joining of the N- and C-termini resulted in several cyclic α -CTx MII analogs. Two of these analogs had structures similar to native α -CTx MII, although only one of these analogs—the one with the longest linker—retained activity similar to native α -CTx MII. The cyclic analog was much more stable than the native toxin in human plasma^[84]. To enhance the lipophilicity of α -CTx MII, the toxin was conjugated with 2-amino-*D,L*-dodecanoic acid (Laa) at the N-terminus. This terminally-conjugated α -CTx MII analog had similar structure and activity compared to the parent peptide^[85], but displayed significantly improved permeability across Caco-2 cell monolayers^[85]. Although the Laa-conjugated analog did not cross the blood brain barrier to any great extent, its absorption through the GI tract after oral administration was greater than the parent α -CTx MII^[86].

A number of other α -conotoxins have selectivity either towards $\alpha 3\beta 2$ or $\alpha 6\beta 2\beta 3$ nAChRs. α -CTx PIA, discovered through PCR-based cloning of cDNA from venom duct of *Conus purpurascens*, has about 70-fold lower IC₅₀ for chimeric

Table 4. Some physiological functions mediated by nAChRs and the subtype(s) involved, as determined by pharmacological characterization using the indicated α -conotoxin (s).

Physiological function	nAChR involved	α -Conotoxin(s) used	Reference
Striatal DA release	$\alpha 6\beta 2, \alpha 6\beta 2\beta 3, \alpha 6\alpha 4\beta 2^*$	MII, PIA, MII[E11A]	[55-67, 70]
Hippocampal NE release-Rat	$\alpha 3\beta 4$	AuIB	[72]
Hippocampal NE release-Mouse	$\alpha 6\alpha 4\beta 2\beta 3, \alpha 6\alpha 4\beta 2\beta 4\beta 3$	MII, BuIA, PIA	[73]
Food/alcohol reinforcement	$\alpha 3\beta 2, \alpha 6^*$	MII	[132-135]
Parkinson's disease	$\alpha 6\alpha 4\beta 2^*$	MII[E11A]	[83]
Pain/inflammatory response	$\alpha 9\alpha 10$	Vc1.1, RgIA	[116, 118], but see [117, 120]

$\alpha 6/\alpha 3\beta 2\beta 3$ vs $\alpha 3\beta 2$ nAChRs^[87]. In contrast, α -CTx OmIA, purified from venom of *Conus omaria*, has a preferential (20-fold) selectivity for $\alpha 3\beta 2$ vs $\alpha 6/\alpha 3\beta 2\beta 3$ nAChRs^[88,89]. It is also a potent inhibitor of $\alpha 7$ nAChR^[89].

α -Conotoxins GIC and GID Two α -conotoxins specific for neuronal nAChRs have been purified from the venom of *Conus geographus*. α -CTx GIC, an $\alpha 4/7$ conotoxin identified from the genomic DNA of *Conus geographus*, has low nanomolar potency for $\alpha 3\beta 2$ and $\alpha 6\beta 2$ nAChRs vs muscle, $\alpha 3\beta 4$ and $\alpha 4\beta 2$ nAChRs^[90,91]. α -CTx GID, although similar to α -CTx GIC in its inter-cysteine loop spacing ($\alpha 4/7$), is structurally different from α -CTx GIC and other neuronal nAChR targeted α -conotoxins in several ways. First, it has additional N-terminal residues compared to α -CTx GIC and most other previously identified α -conotoxins. Second, it has two posttranslational modifications: a γ -carboxyglutamic acid residue just before the first Cys residue and a hydroxyproline at position 16. Third, there is a positively charged residue, Arg, at position 12, whereas α -CTx GIC and most other α -conotoxins have either a hydrophobic (Ala or Phe) or uncharged (Asn) residue. Interestingly, a positive charge at this position seems to be responsible for α -CTx GID's relatively high affinity for $\alpha 4\beta 2$ nAChRs compared to other α -conotoxins lacking this positive residue^[92,93]. It is worth noting here that the two muscle-specific α -conotoxins-SrIA and SrIB—that have potentiating effects on the $\alpha 4\beta 2$ nAChR also have an Arg at the homologous position (Table 1). An 'Ala walk' of α -CTx GID also indicates important roles for most residues of the toxin, with the exception of Val13, in interaction with $\alpha 4\beta 2$ nAChR^[93]. Similar to α -CTx GIC, α -CTx GID also potently blocks $\alpha 3\beta 2$ nAChR^[92,93]. In contrast to α -CTx GIC, however, α -CTx GID also blocks $\alpha 7$ nAChR^[92,93]. Structure-activity studies indicate the most important residue for interaction with both the $\alpha 7$ and the $\alpha 3\beta 2$ nAChRs is Pro9, with smaller contribution by Asp3 and Arg12. Asn14 seems to be important for interaction with $\alpha 7$ nAChR only^[93]. The $\beta 2$ subunit residues conferring the high potency of α -CTx GID on $\alpha 3\beta 2$ nAChR are the same residues that also confer high potency of α -CTx MII and α -CTx PnIA for this receptor subtype^[75].

α -Conotoxin Epl α -CTx Epl, an $\alpha 4/7$ -conotoxin purified from the venom of *Conus episcopatus*, is another example of a post-translationally modified conotoxin. This conotoxin is different from previously characterized α -conotoxins in that it has a sulfated Tyr at position 15^[94]. However, both the native sulfated and synthetic non-sulfated peptide are similar in activity and inhibit α -bungarotoxin-resistant nAChR responses in adrenal chromaffin cells and rat intracardiac ganglia parasympathetic neurons, but do not inhibit muscle

nAChR responses^[94]. Therefore, this toxin was designated as a specific blocker of $\alpha 3\beta 2$ and $\alpha 3\beta 4$, but not $\alpha 7$, nAChRs. However, studies with heterologously expressed nAChRs in oocytes showed the inverse selectivity, potent inhibition of $\alpha 7$ nAChRs with little effect on $\alpha 3\beta 4$ or $\alpha 3\beta 2$ nAChRs^[95]. The inhibition of $\alpha 7$ nAChRs by α -CTx Epl is not surprising considering that α -CTx Epl is identical to α -CTx ImI in its first loop, the region containing residues shown to be important for interaction of α -CTx ImI with $\alpha 7$ nAChRs^[41].

α -Conotoxins AnIA, AnIB, and AnIC Three peptides isolated from the venom of *Conus anemone* add to the list of sulfated α -conotoxins^[96]. α -CTx AnIA, α -CTx AnIB, and α -CTx AnIC all have a sulfotyrosine at position 16. The synthetic α -CTx AnIB had subnanomolar potency at $\alpha 3\beta 2$ nAChRs, with about 200-fold lower potency at $\alpha 7$ nAChR, and little or no activity on muscle and other tested heteromeric nAChRs. The non-sulfated peptide retained activity at $\alpha 3\beta 2$ nAChRs, whereas it was 10-fold less potent on $\alpha 7$ nAChR. Removal of the two N-terminal glycines (to yield the same toxin as α -CTx AnIA) negatively affected binding kinetics, and as a result potency, of the toxin on $\alpha 3\beta 2$ nAChRs^[96].

α -Conotoxins AuIA, AuIB, and AuIC Three α -conotoxins isolated from the venom of *Conus aulicus* are α -CTx AuIA, AuIB, and AuIC. Similar to most other neuronal nAChR blocking α -conotoxins, α -CTx AuIA and AuIC belong to the $\alpha 4/7$ family, whereas α -CTx AuIB has the unusual $4/6$ inter-cysteine loop spacing^[72]. All three are selective blockers of the neuronal subtype, $\alpha 3\beta 4$, with α -CTx AuIB being the most potent^[72]. This α -conotoxin has been used to show involvement of distinct nAChR subtypes in modulation of [³H]NE, [³H]ACh, and [³H]DA release from hippocampal, interpeduncular nucleus and striatal synaptosomes, respectively^[72,97].

α -Conotoxins PnIA and PnIB α -CTx PnIA and PnIB were purified from the venom of *Conus pennaceus*^[98]. Both toxins have a post-translational modification, sulfotyrosine at position 15^[99]; however, the functional characterizations were all performed with non-sulfated synthetic toxins. Although they only differ in two amino acids, α -CTx PnIA is a potent blocker of $\alpha 3\beta 2$ nAChR, whereas α -CTx PnIB blocks $\alpha 7$ nAChR more potently^[100].

Substitution of Ala10 in α -CTx PnIA with Leu, found in the homologous position in α -CTx PnIB, shifts selectivity of α -CTx PnIA[A10L] towards $\alpha 7$, with a potency that is even greater than α -CTx PnIB^[100,101]. The systematic truncation of the second loop affects the potency of α -CTx PnIA[A10L] for both the $\alpha 7$ nAChR and AChBP due to the loss of several hydrogen bonds between toxin and receptor upon

toxin truncation^[102]. Replacing Asn11 with Ser in α -CTx PnIA, the other residue different between α -CTx PnIA and α -CTx PnIB, caused loss of potency for both $\alpha 3\beta 2$ and $\alpha 7$ nAChRs^[100].

Structure-activity studies have located residues in the $\alpha 3$ subunit that confer affinity for α -CTx PnIA. These include Pro182, Ile188 (also found to interact with α -CTx MII) and Gln198^[103]. All three residues were found to be located within the C-loop of the subunit. The $\beta 2$ subunit residues that interact with α -CTx PnIA are the same residues that also interact with α -CTx MII and α -CTx GID^[75]. Adding an additional positive charge to the C-terminus of α -CTx PnIA[A10L] to yield the analog α -CTx PnIA[A10L, D14K] enhanced the affinity of the toxin for *Lymnaea* and *Aplysia* AChBPs^[33, 104]. A recent study presented the crystal structure of α -CTx PnIA[A10L;D14K] bound to *Aplysia* AChBP and indicated predominantly hydrophobic and hydrophobic/aromatic interactions between the analog and the AChBP binding pocket^[104]. Double cycle mutant analysis has also indicated pairwise hydrophobic and aromatic interactions between α -CTx PnIB and the $\alpha 7$ nAChR^[105].

α -Conotoxin BuIA α -CTx BuIA was cloned from RNA extracted from the venom of *Conus bullatus*^[106]. Similar to the muscle nAChR blocking α -CTx PIB, it possesses a 4/4 cysteine loop spacing. Unlike other α -conotoxins, α -CTx BuIA is not specific for a particular subtype of nAChR, blocking almost all neuronal subtypes with nanomolar potency, with exception of $\alpha 4\beta 2$ ^[106]. However, its differential kinetics can distinguish between nAChRs that contain either a $\beta 2$ or a $\beta 4$ subunit: block of $\beta 2^*$ nAChRs is rapidly reversed whereas block of $\beta 4^*$ nAChRs is only slowly reversed upon toxin washout^[106]. The subunit residues that are critical to these off-rate differences have been determined and, interestingly, are the same residues that interact with a number of other α -conotoxins^[75, 107]. Two studies have solved the structure of α -CTx BuIA^[108, 109]. The latter study has shown that the native globular structure of α -CTx BuIA is highly flexible and maintains multiple conformations in solution, as opposed to some other α -conotoxins that have a rigid globular structure^[109]. This multiple-conformation structural feature may underlie the toxin's promiscuous selectivity profile.

The differential kinetics of α -CTx BuIA were utilized to determine the extent of the participation of the $\beta 2$ and the $\beta 4$ subunits in nAChRs on rat and mouse hippocampal noradrenergic terminals. These studies indicated the presence of the $\beta 4$ subunit in all nAChRs on rat terminals, but its presence in only 60% of mouse terminal nAChRs^[73].

α -Conotoxins PeIA, RgIA and Vc1.1 Three newly discovered α -conotoxins have been shown to target the $\alpha 9\alpha 10$

nAChR, a subtype with highly unusual pharmacology as compared to other nAChRs^[110, 111]. α -CTx PeIA, cloned from the venom of *Conus pergrandis*, blocks heterologously expressed $\alpha 9\alpha 10$ nAChRs, as well as native $\alpha 9\alpha 10$ nAChRs in cochlear hair cells with IC_{50} s of about 7 and 4 nmol/L, respectively^[112]. However, this toxin is also a potent blocker of $\alpha 3\beta 2$ and chimeric $\alpha 6/\alpha 3\beta 2\beta 3$ nAChRs, with IC_{50} s of about 23 and 30 nmol/L, respectively^[112].

α -CTx RgIA, cloned from the venom of marine worm-hunting species *Conus regius*, is another $\alpha 4/3$ conotoxin similar to α -CTx Iml (Figure 1); however, unlike α -CTx Iml, it's a much more potent blocker of $\alpha 9\alpha 10$ than $\alpha 7$ nAChRs^[113] and the most selective $\alpha 9\alpha 10$ antagonist reported to date. α -CTx Vc1.1, cloned from the venom of *Conus victoriae*, is an $\alpha 4/7$ conotoxin originally shown to block nAChRs found in adrenal chromaffin cells^[114, 115]. In addition, this peptide blocks vascular inflammatory responses evoked by electrical stimulation of unmyelinated sensory nerves^[114]. Further pharmacological characterization of this α -conotoxin indicated that it is a potent inhibitor of the $\alpha 9\alpha 10$ nAChR^[116, 117]. Unlike α -CTx RgIA, however, native α -CTx Vc1.1 (referred to as Vc1a) contains three post-translational modifications: a hydroxyproline at position 6, a γ -carboxyglutamate at position 14 and an amidated C-terminus^[117]. Both α -CTx RgIA and α -CTx Vc1.1 were shown to be effective analgesic agents in a rat model of nerve injury^[116-118] and α -CTx Vc1.1 (drug name ACV1) entered human phase II clinical trials for treatment of neuropathic pain^[119]. However, the role of the $\alpha 9\alpha 10$ nAChRs in mediating this analgesic effect has been challenged^[117, 120].

Structure-activity data with α -CTx RgIA have indicated that this toxin binds to the ACh binding site on the receptor and that toxin residues Asp5, Pro6, Arg7, and Arg9 are important for interaction with $\alpha 9\alpha 10$ nAChRs^[121]. The recently published three-dimensional structure of α -CTx RgIA also confirms an important role for Arg9 in interacting with negatively charged residues in the $\alpha 9\alpha 10$ nAChR^[122]. Saturation transfer difference NMR studies examining the binding of α -CTx Vc1.1 to *Lymnaea stagnalis* AChBP, a close structural homolog of the $\alpha 7$ nAChR binding site, indicates a role for Tyr10 in binding to the AChBP^[123]. Interestingly, this residue in α -CTx RgIA does not seem to be important for interaction with $\alpha 9\alpha 10$ nAChRs^[121].

α -Conotoxin TxIA α -CTx TxIA, from the venom of *Conus textile*, was recently discovered using a novel approach for identification of new α -conotoxins from crude venom^[124]. In this approach, the venom of *Conus textile* was screened against the *Lymnaea* AChBP in a competition binding assay with ¹²⁵I- α -bungarotoxin. Biochemical characterization indi-

cated that α -CTx TxIA belongs to the α 4/7 conotoxin family and has the same cysteine arrangement and disulfide connectivity common to other α -conotoxins in this family. Binding and functional assays indicated that the affinity of this toxin for *Lymnaea* AChBP (1.7 nmol/L) was higher than other previously identified α -conotoxins, and that α -CTx TxIA had high potency for α 3 β 2 nAChRs. Structure-activity studies, together with co-crystallization of an analog of α -CTxIA, α -CTx TxIA[A10L], with *Aplysia* AChBP indicated an important role for a long chain hydrophobic residue at position 9 or 10 and the Arg at position 5 for toxin affinity for AChBP and α 7, but not α 3 β 2, nAChRs^[124]

α -Conotoxin Lp1.1 α -CTx Lp1.1 was cloned from both the genomic DNA and cDNA of *Conus leopardus*^[125]. Although it belongs to the α 4/7 conotoxin family, its primary sequence is unique in that it lacks the conserved Ser and Pro that is found in the first loop of all known neuronally active α -conotoxins (Table 2). This toxin caused uncoordinated swimming when injected intramuscularly in fish. At higher concentrations, it causes seizure and paralysis^[126]. Interestingly, another α 4/7 conotoxin (LeD2) isolated from a different *Conus* species, *Conus litteratus*, has the identical sequence to Lp1.1^[127].

α -Conotoxins ArIA and ArIB We recently identified two new α -conotoxins from the venom of *Conus arenatus*, α -CTx ArIA and α -CTx ArIB^[128]. Both belong to the α 4/7 conotoxin family and are potent blockers of the α 7 nAChR. However, both toxins also block the α 3 β 2 nAChR with nanomolar potency^[128]. Structure-function analysis was used to create two analogs of α -CTx ArIB, α -CTx ArIB[V11L; V16A] and α -CTx ArIB[V11L; V16D], which have high affinity for α 7 nAChRs but have comparatively low activity on α 3 β 2 nAChRs^[128]. Compared to α -bungarotoxin, however, the faster off-rate kinetics of the α -CTx ArIB analogs make them useful ligands in equilibrium binding experiments. α -CTx ArIB[V11L; V16D] blocks rat, mouse and human nAChRs^[128, 129]. A radiolabeled version, ¹²⁵I- α -CTx ArIB[V11L; V16A], has also been developed^[130].

Concluding remarks

The last quarter of the century has witnessed the discovery of variety of different α -conotoxins targeting various isoforms of nAChRs. The next few years promise even more groundbreaking progress thanks to the recent advancements in phylogeny and exogenomic discovery of novel conotoxins^[6, 131]. In addition, structure-activity studies in combination with homology modeling will lead to better understanding of interactions between α -conotoxins and nAChR ligand

binding site, allowing the creation of analogs with improved potency and/or selectivity towards particular subtypes of nAChRs. In view of the important physiological role of nAChRs in pain, inflammation, nicotine addiction, Alzheimer's and Parkinson's disease, specific targeting of the relevant nAChR subtypes is an attractive pharmaceutical strategy, with the α -conotoxins being among the most promising drug development leads.

Acknowledgements

We thank Dr Baldomero M OLIVERA for providing comments on the manuscript. Support provided by Kirschstein-National Research Service Award Postdoctoral Fellowship DA 016835 (to Layla AZAM) and by National Institutes of Health Grant MH53631 (to J Michael MCINTOSH).

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