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Addendum

# Structural Determinants for α-Neurotoxin Sensitivity in Muscle nAChR and Their Implications for the Gating Mechanism

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Addendum to:

*Crystal Structure of the Extracellular Domain of nAChR α1 Bound to α-Bungarotoxin at 1.94 A Resolution.*

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## ABSTRACT

Neurotoxins from snake venoms act as potent antagonists on the nicotinic acetylcholine receptors (nAChRs). Alpha-neurotoxins such as α-bungarotoxin (α-Btx) selectively bind to the skeletal muscle nAChRs among other subtypes, causing failure of the neuromuscular transmission. Through evolution, some species including snakes and mongoose have developed resistance to α-neurotoxins via specific amino acid substitutions in their muscle-type nAChR α1 subunit, which constitutes most of the toxin-binding site. Here we analyze these sequence variations in the context of our recent crystal structure of the extracellular domain of the mouse nAChR α1 bound to α-Btx. Our structure suggests that α-Btx has evolved as an extremely potent antagonist of muscle nAChR by binding the receptor tightly, blocking its ligand site, and locking its conformation in a closed state. Conversely, most toxin-resistant mutations occur at the α-Btx binding interface on nAChR α1 but away from the agonist binding site. These mutations can interfere with the binding of α-Btx without having deleterious effect on the gating function. These analyses not only help understand the structural determinants for neurotoxin sensitivity in muscle-type nAChR, but also shed light on its gating mechanism.

The nicotinic acetylcholine receptors (nAChRs) are the prototypic members of the Cys-loop family of ligand-gated ion channels that also includes serotonin (5-$HT_3$), glycine and γ-aminobutyric acid ($GABA_{A,C}$) receptors.[1-3] nAChRs play a fundamental role in the central and peripheral nervous system as they mediate the fast chemical transmission of the electric signal at the synaptic cleft and at the neuromuscular junction.[1]

Upon binding to agonists like acetylcholine (ACh), nAChRs undergo a conformational change that results in the opening of the ion-selective transmembrane pore.[2-4] Alpha-neurotoxins present in snake and snail venoms are typical antagonists, inhibiting with different potency the receptors function. For example, α-Bungarotoxin (α-Btx) from *Bungarus multicinctus* binds to the neuronal nAChR homo-$\alpha_7$-pentamer, and to the muscle nAChR hetero-pentamer, causing the channel blockage, which eventually results in paralysis and respiratory failure.[5,6]

Intriguingly, in vivo and in vitro studies showed that the effects of α-Btx and other α-neurotoxins vary throughout animal species, from lethality upon low-dose exposure in mice and rabbits, to resistance to medium-dose exposure in hedgehogs, and to near-insensitivity even at high-dose exposure in snakes and mongooses.[7,8] High resistance to toxin was shown not to be due to neutralizing factors in the animals' blood serum, but rather to specific features of the animals' nAChRs.[7,9] Furthermore, two studies showed that the α1 subunit of the muscle-type nAChR bears the major structural determinants for α-neurotoxins sensitivity.[10,11] This is consistent with the fact that most of the structural elements constituting the antagonist-binding site in nAChRs are located in the α subunits.[3,4]

Ion channels are major pharmaceutical targets,[12] and nAChRs are of particular relevance to a variety of diseases including Parkinson's, Alzheimer's, myasthenia gravis, schizophrenia, epilepsy, depression and substance addiction.[5] As natural antagonists of nAChRs, α-neurotoxins may serve as a template for drugs designed for specialized treatment of neurological diseases, as well as analgesics and muscle relaxants.[1,5,6] In this sense, knowledge of the structural features that determine the binding and potency of different α-neurotoxins on targeted receptors may greatly improve the efficacy of neurotoxin-based drug design.[6]

We have recently solved the crystal structure of the extracellular domain of the mouse nAChR α1 subunit (residues 1–211; α211 henceforth) in complex with α-Btx at 1.94Å resolution (Fig. 1A, pdb code 2QC1).[4] The structure reveals a number of channel-specific

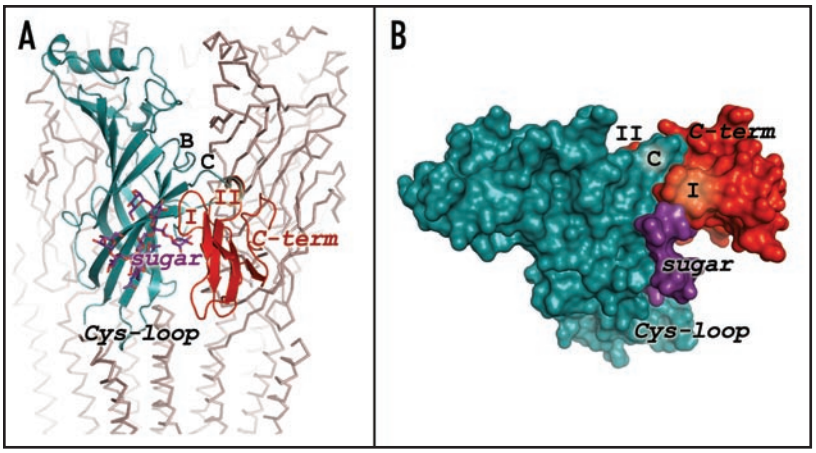


Figure 1. Overall structure of the mouse nAChR α1 extracellular domain (ECD, α211) bound to α-Btx. The nAChR α1 ECD is colored in cyan, α-Btx in orange, and carbohydrate in magenta. This color scheme is used through out the illustration unless otherwise stated. (A) A view of the α211/α-Btx complex in the context of the intact nAChR pentamer. The β-sandwich core of α211 (in ribbon) is superimposed on the ECD of the *Torpedo* nAChR α subunit (pdb code 2BG9).[18] For clarity, the original *Torpedo* nAChR α ECD is not shown, whereas the rest of the receptor is shown in Cα trace. It is clear from this picture that α-Btx (in ribbon) is located between two subunits and that the carbohydrate chain (in stick model) binds to the outer sheet of the α ECD. (B) Surface representation of the α211/α-Btx complex showing the extensive and intimate interaction between the receptor, sugar and the toxin. Loop B, Loop C, the Cys-loop and the sugar chain of the receptor, finger I, finger II and the C-terminal loop of the toxin, are labeled to provide spatial references.

features absent in the non-channel homologue ACh-binding protein (AChBP).[4,13] Most notably, a hydrophilic cavity conserved in nAChR but absent in AChBP was found inside the β-sandwich core of nAChR α1, which is shown to be essential for channel gating by mutagenesis and electrophysiology studies.[4] The structure also reveals the atomic details of an N-linked glycan conserved in muscle-type nAChR, which anchors on the membrane-facing Cys-loop and branches upward to the back side of loop C, thus linking the two functionally important loops together. The upper end of the sugar chain makes extensive contacts to finger I of α-Btx, which seems to prevent the glycan chain to reach further to the tip of loop C. Consistent with these structural features, biochemical analyses showed that the glycan chain plays crucial roles in channel gating and toxin binding.[4]

Our structure provides an unprecedented view of the α211/α-Btx interactions at the atomic level. Finger I, finger II and the *C*-terminal loop of the toxin, and loop A, loop B, loop C and the carbohydrate chain from the receptor form the major part of the binding interface, which buries ~1,800Å$^2$ of solvent-accessible area and displays exquisite shape and chemical complementarity (Fig. 1B). The interactions involve numerous main chain contacts that give rise to an unusually tight protein-protein binding interface. Two key residues in toxin finger II, Arg36 and Phe32, insert into the receptor aromatic cage (α1 residues Tyr93, Tyr190, Tyr198 and Trp149), blocking the access of agonists to the active site. Thus, it seems that the toxin exerts several effects on the gating function of the muscle nAChR. One is to block the access of the agonist-binding site. The other is to potentially disrupt the sugar-mediated communication between the Cys-loop and loop C. Finally, the toxin may also modulate the structure of the Cys-loop through interactions with the glycan chain, which in turn may affect the gating of the transmembrane pore.

It is expected that toxin-resistant mutations will likely affect the binding of α-neurotoxins. As revealed by our crystal structure,[4] most of the sequence variations implicated in modulating the receptor's sensitivity to α-neurotoxins are indeed located at or near the receptor/toxin interface[7-11,14,15] (Fig. 2A and B). Three positions in the α1 subunit, 187, 189 and 194, appear to be particularly important. While all toxin-sensitive animals have two aromatic residues (Trp187 and Phe189 in mouse) and a proline (Pro194), snakes and mongoose have both aromatic residues replaced by polar residues, and Pro194 by a more flexible leucine residue (Fig. 2A). Additionally, substitutions of Pro197His, Gly153Ser and Tyr112His in mongoose, and Lys185Trp in snakes, also seem to cosegregate to various degrees with toxin resistance.[15] Animals with a medium toxin resistance have replacements only at the two aromatic positions 187 and 189 (Fig. 2A).

As our structure shows, these amino acid changes occur away from the aromatic cage that is involved in agonist binding, consistent with the observation that the muscle-type nAChRs from toxin-resistant species have similar functional properties to those from the toxin-sensitive animals.[10,11] However, as discussed below, most of these changes, except for Tyr112His in mongoose, are located at positions that affect α-Btx binding either directly or indirectly (Fig. 2B).

Phe189 is located at the center of the toxin/receptor interface, implicating its critical role in toxin binding. Its aromatic side chain inserts into a hydrophobic groove formed by finger I, finger II and the C-terminal loop of α-Btx (Fig. 2C). The side chain conformation of Phe189 is stabilized partly by the pyrrolidine ring of Pro197, which in turns packs against the main chain of Gly153 (Fig. 2C). In snake and mongoose, position 189 is occupied by asparagine (Asn189) and threonine (Thr189), respectively. These hydrophilic residues will not interact favorably with the hydrophobic groove on α-Btx. Substitutions on the adjacent residues, such as Pro197His and Gly153Ser in mongoose, may further weaken toxin binding indirectly through modulating the side chain conformation of Phe189. Gly153Ser may also alter the main chain conformation of loop B and affect the receptor's binding affinity for acetylcholine.[16] In snakes, Asn189 constitutes a functional glycosylation site that introduces a bulky glycan right at the center of the toxin/receptor interface,[7,8,11] which seems to be the most effective way to block toxin binding through steric clashes.

Trp187 makes little direct contact with the toxin but interacts with the carbohydrate chain extensively. These interactions apparently stabilize the structure of the carbohydrate and facilitate its interaction with finger I of the toxin (Fig. 2D). We have shown that the carbohydrate chain is important for α-Btx binding to the receptor.[4] Thus Trp187 may contribute to toxin binding indirectly through the carbohydrate. In snake and mongoose, position 187 is occupied by Ser (Ser187) and Asn (Asn187), respectively. These substitutions may alter the local sugar conformation leading to diminished interactions between α-Btx and the receptor (Fig. 2D). In the α211/α-Btx complex structure, the aliphatic side chain of Lys185 packs against the aromatic ring of Trp187, the Lys185Trp substitution in snakes may therefore contribute to toxin resistance by altering the conformation of Trp187 and/or the sugar chain.

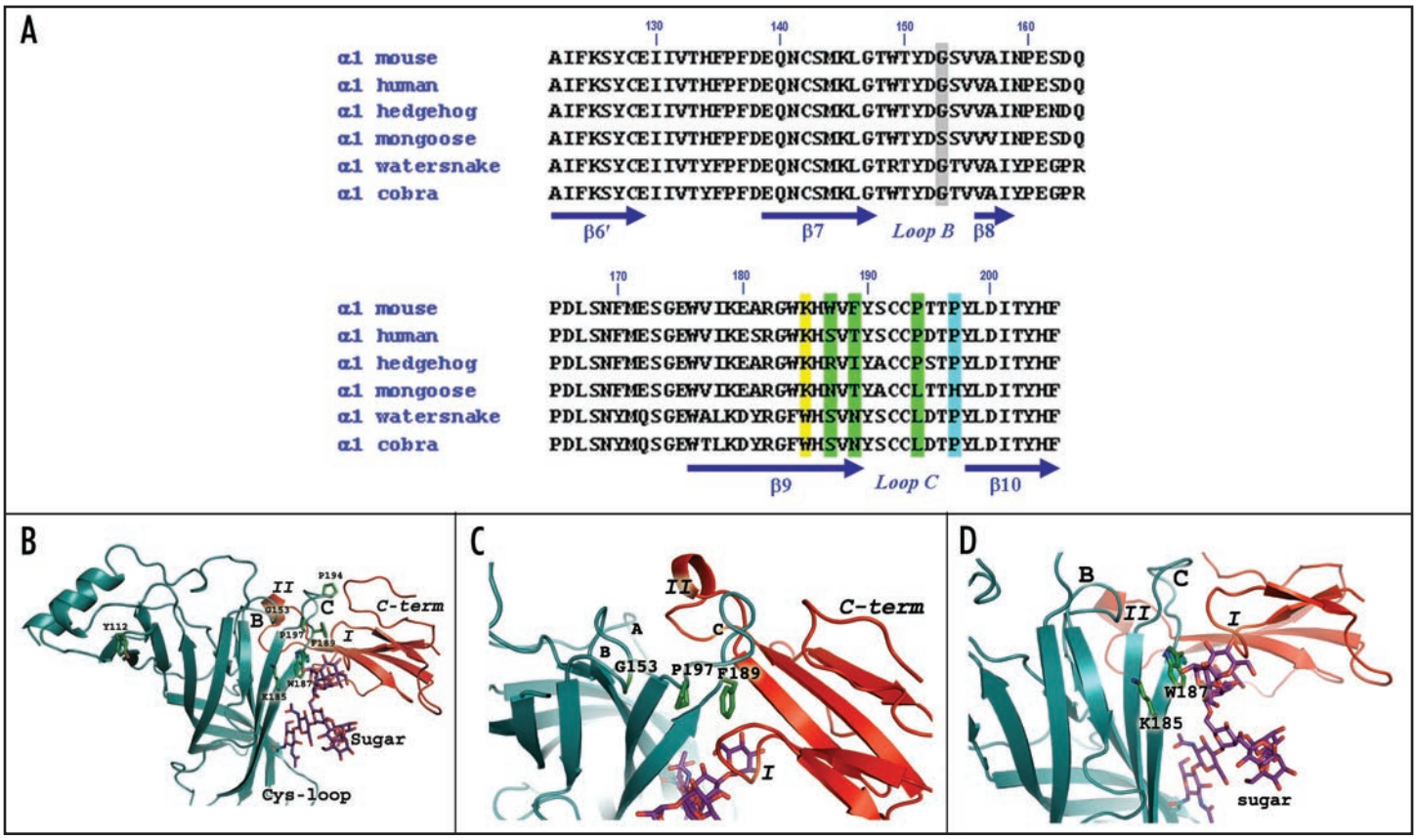


Figure 2. Structural determinants for α-neurotoxin sensitivity in muscle nAChR (A) partial sequence alignment of nAChR α1 subunit from representative toxin sensitive and resistance species. The three key positions (187, 189 and 194) are highlighted in green. The other three positions discussed in the text (153, 185 and 197) are colored in gray, yellow and blue, respectively. (B) Sequence variations implicated in modulating muscle nAChR's sensitivity to α-neurotoxin are mapped on the three-dimensional structure of the α211/α-Btx complex, viewed in an orientation similar to that of Figure 1B. The amino acid residues involved are shown in stick model and colored in green. (C) A zoom-in view of Phe189 and its surrounding region as discussed in the text. (D) A zoom-in view of Trp187 and its surrounding region as discussed in the text.

Interestingly, mongoose-Asn187 also constitutes a glycosylation site that was shown to be actually glycosylated.[14] The glycan chain may interfere with toxin binding. However, since residue 187 is located at the periphery of the toxin/receptor interface, a glycan chain introduced at this site may not be as effective in blocking toxin binding as that found in snakes at position 189.[11,14,15] It is important to note that while the conserved glycan on the Cys-loop contributes positively to toxin binding, the glycans introduced at Asn189 in snakes and Asn187 in mongoose likely interfere with toxin binding due to steric clashes.

In the α211/α-Btx structure, Pro194 packs in a small hydrophobic pocket formed by α-Btx residues His68, Lys70 and Gln71 (not shown). Pro194 also confers to the upper tip of α1 loop C a peculiar conformation to which the *C*-terminal loop of α-Btx (residues 67 to 71) perfectly complements (Fig. 2B). These structural features suggest that Pro194 is likely important for α-Btx binding and its substitutions contribute to toxin resistance.

Overall, our studies of the α211/α-Btx complex provide structural rationale for most amino acid substitutions implicated in modulating muscle nAChR's sensitivity to α-neurotoxins. Some substitutions, such as Tyr112His in mongoose if relevant to toxin resistance, cannot be explained by our structure. It seems that the most effective strategy for toxin resistance, as employed by snakes, is to introduce a glycan through Asn189 at the center of the toxin/receptor interface. Asn187 in mongoose may also work similarly. In the absence of added glycosylation, partial toxin resistance in humans and hedgehogs may be explained by substituting aromatic residues at positions 187 and 189 with non-aromatic residues.

Our analyses here suggest once again that glycosylation on nAChR can have significant functional consequences beyond folding and trafficking.[4,17] We have previously shown that the glycan chain attached to the Cys-loop is required for the gating function of muscle nAChR, presumably by providing a movable linker between the ligand-binding loop (Loop C) and the membrane interaction loop (the Cys-loop). Here in toxin-resistant nAChRs, glycosylation at specific sites has been employed as a strategy to block toxin binding. It is possible that the newly introduced glycans may even interact with the glycan chain emanating from the Cys-loop thus contributing to the communication between the ligand-binding site and the membrane pore.

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