

Cobra (*Naja* spp.) Nicotinic Acetylcholine Receptor Exhibits Resistance to Erabu Sea Snake (*Laticauda semifasciata*) Short-Chain α -Neurotoxin

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Abstract. Snake α -neurotoxins of Elapidae venoms are grouped into two structural classes, short-chain and long-chain α -neurotoxins. While these two classes share many chemical and biological characteristics, there are also distinct dissimilarities between them, including their binding site on the nicotinic acetylcholine receptor (nAChR), specificity among species of Chordata, and the associated pharmacological effects. In the present study we test the hypothesis that structural motifs that evolved to confer natural resistance against conspecific long-chain α -neurotoxins in Elapidae snakes also interfere with the biological action of short-chain α -neurotoxins. We expressed functional nAChRs that contains segments or single residues of the Elapidae nAChR ligand binding domain and tested the effect of short-chain α -neurotoxin erabutoxin-a (ETX-a) from the Erabu sea snake *Laticauda semifasciata* on the acetylcholine-induced currents as measured by two-microelectrode voltage clamp. Our results show that the Elapidae nAChR α subunit segment T¹⁵⁴–L²⁰⁸ ligand binding domain has an inhibitory effect on the pharmacological action of ETX-a. This effect is primarily attributed to the presence of glycosylation at position N¹⁸⁹. If the gly-

cosylation is removed from the T¹⁵⁴–L²⁰⁸ segment, the nAChR will be inhibited, however, to a lesser extent than seen in the mouse. This effect correlates with the variations in α -neurotoxin sensitivity of different species and, importantly, reflects the evolutionary conservation of the binding site on the nAChR polypeptide backbone per se. Phylogenetic analysis of α -neurotoxin resistance suggests that α -neurotoxin-resistant nAChR evolved first, which permitted the evolution of snake venom α -neurotoxins. A model describing α -neurotoxin resistance in Elapidae snakes is presented.

Key words: *Laticauda semifasciata* — Hydrophiinae — Elapidae — Erabutoxin-a — Short-chain snake α -neurotoxin — Nicotinic acetylcholine receptor — Natural resistance

Introduction

Snake α -neurotoxins are the major lethal components of Elapidae (including, for example, cobras, kraits, sea kraits, and true sea snakes [Slowinski and Keogh 2000]) venoms and they play a fundamental role in the snake feeding mechanism and defensive strategy. The common target of α -neurotoxins is the muscle-type nicotinic acetylcholine receptor (nAChR), a ligand-gated ion channel on the postsynaptic fold of the neuromuscular junction with the subunit stoichiometry of $\alpha_2\beta\gamma\delta$ (Karlín 2002; Sine 2002). Upon binding to the nAChR, α -neurotoxin prevents the binding of

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the natural ligand acetylcholine (ACh) and the subsequent ACh induced ion flow, resulting in a neuromuscular inhibition of the target species.

α -Neurotoxins, based on their molecular structures, can be classified into two classes, termed short-chain and long-chain α -neurotoxins. Short-chain α -neurotoxins contain 60–62 amino acid residues and four disulfide bridges. Erabutoxin-a (ETX-a) isolated from the venom of Erabu sea snake, *Laticauda semifasciata*, Elapidae (Sato and Tamiya 1971) is a representative member of short-chain α -neurotoxins. The crystal structure of its close relative, erabutoxin-b (ETX-b), from the same venom has been determined (Low et al. 1976; Tsernoglou and Petsko 1976). The polypeptide chain is folded into three large main-chain loops bound by four disulfide bridges close to one end, with an overall shape of thin, flat disk. ETX-b fits into an ellipsoidal envelope approximately $38 \times 28 \times 15 \text{ \AA}$ (Low et al. 1971).

Long-chain α -neurotoxins consist of 66–74 amino acid residues and usually have a fifth disulfide bridge in addition to the four disulfide bridges present in their short-chain counterparts. In comparison with short-chain α -neurotoxins, long-chain α -neurotoxins have further sequence characteristics of distinct deletions and insertions of amino acids, as well as the presence of an extra C-terminal segment of five to nine amino acid residues (Endo and Tamiya 1991). α -Bungarotoxin (α -BTX) isolated from the venom of banded krait, *Bungarus multicinctus*, Elapidae (Lee and Chang 1966; Mebs et al. 1972) is a representative member of long-chain α -neurotoxin group. The crystal structure of α -BTX has been solved (Agard and Stroud 1982; Stroud 1982; Love and Stroud 1986), and the overall arrangement of polypeptide chain folding is similar to that found in ETX-b. The main differences are the extra tail segment of long-chain α -neurotoxins and the first main-chain loop. The overall dimensions of α -BTX are $40 \times 30 \times 20 \text{ \AA}$ (Love and Stroud 1986). Both classes of α -neurotoxins belong to the three-finger toxin family. Despite the structural conservation within this family, members exhibit diverse biological function (Fry et al. 2003).

The binding site of short-chain α -neurotoxins on the nAChR is less well characterized compared to long-chain α -neurotoxins (Weber and Changeux 1974; Chicheportiche et al. 1975). In general, it is regarded that short-chain and long-chain α -neurotoxins share a similar overall topology in their interaction with the nAChR (Teixeira-Clerc et al. 2002). However, the neuromuscular preparations of some reptile (Reptilia) species, including *Cordylus jonesi* (Cordylidae, Sauria), *Lacerta* sp. (Lacertidae, Sauria), and *Epicrates cenchria* (Boidae, Serpentes), are blocked by α -BTX but not by the short-chain α -

neurotoxin, α -atratoxin from *Naja atra* (Burden et al. 1975), suggesting that their binding mechanism must be different. Monoclonal antibodies raised against the long-chain α -neurotoxin, α -cobratoxin of *N. n. siamensis* (overlaps the nAChR binding site), that recognize four other long-chain α -neurotoxin members fail to recognize short-chain α -neurotoxins (Charpentier 1990). Furthermore, in contrast to α -BTX, short-chain α -neurotoxins, including ETX-a and cobratoxin from *Naja atra*, fail to recognize the peptide segment R¹⁸²–Y¹⁹⁸ from the α subunit of *Torpedo* and *Homo* nAChR (Ruan et al. 1991). Similarly, *Torpedo* nAChR α subunit segment K¹⁸⁵–L¹⁹⁹ binds the short-chain *Naja nigricollis* toxin α with substantially less affinity than the long-chain α -cobratoxin of *N. kaouthia* (*N. n. siamensis*) (Fulachier et al. 1994). Binding of *Naja nigricollis* toxin α to *Torpedo* nAChR analyzed by resonance Raman spectroscopy indicates that α subunit residues W¹⁴⁹, Y¹⁹⁰, C¹⁹², and C¹⁹³ are involved in binding (Negrerie et al. 1991). *N. m. mossambica* I short-chain α -neurotoxin binds to nAChR α subunit D⁹⁹, W¹⁴⁹, V¹⁸⁸, Y¹⁹⁰, Y¹⁹⁸, and D²⁰⁰ (Malany et al. 2000).

Venomous snakes are resistant to the components of conspecific venom. In the case of Elapidae snakes, the resistance against conspecific long-chain α -neurotoxin α -BTX has been shown to be mediated by a unique *N*-glycosylation of the nAChR ligand binding domain (Takacs et al. 2001). If this glycosylation is removed, the nAChR will be inhibited by conspecific long-chain α -neurotoxin, revealing that the binding site of α -BTX is evolutionarily conserved on the nAChR per se (Takacs et al. 2001). Short-chain and long-chain α -neurotoxins have many common characteristics, including molecular structure, pharmacological action, and coexistence in conspecific venoms. Based on these similarities it is possible that the mechanism that protects a snake from the action of long-chain α -neurotoxins also protects against short-chain α -neurotoxins. Furthermore, there are no data available to date describing the molecular mechanisms of natural resistance against short-chain α -neurotoxins or addressing natural resistance to sea snake venom at the molecular level.

In the present study we test the hypothesis that structural motifs that confer resistance against conspecific long-chain α -neurotoxins in Elapidae snakes also interfere with the pharmacological action of short-chain α -neurotoxins. We expressed functional nAChRs that contain segments or single residues of the cobra (*Naja* spp., Elapidae) nAChR and tested the effect of short-chain α -neurotoxin ETX-a on the ACh induced currents by two-microelectrode voltage clamp. We also present a model that describes the α -neurotoxin resistance in Elapidae snakes as well as the evolutionary conservation of snake α -neurotoxin binding site.

Materials and Methods

Materials, Animals, and Toxins

Mus musculus muscle-type nicotinic acetylcholine receptor (nAChR) α , β , γ , and δ subunit cDNA clones in pSP64T vector were kindly provided by Arthur Karlin (Columbia University, New York). DNA restriction enzymes and other DNA modifying enzymes were obtained from New England Biolabs (Beverly, MA) or from Promega (Madison, WI). *Naja haje*, Elapidae (Egyptian cobra), originated from Tanzania and were transported to the United States with the required documentation. *Laticauda semifasciata*, Elapidae (Erabu sea snake), erabutoxin-a (ETX-a) (Sato and Tamiya 1971) was purchased from Sigma (St. Louis, MO). Protein concentrations were determined with the Bradford dye binding assay (Bradford 1976) from Bio-Rad (Richmond, CA) using bovine serum albumin as standard. The GenBank accession numbers for the sequences used in the study are *Mus musculus* (Domestic mouse) muscle-type nicotinic acetylcholine receptor subunits α (X03986), β (M14537), γ (X03818), and δ (K02582) and *Naja haje* (Egyptian cobra) muscle-type nAChR subunit α ligand binding domain (AF077763).

Taxonomy and Nomenclature

Laticauda semifasciata (common names: Erabu sea snake, Philippine sea snake, Broad-band blue sea snake) is a sea krait of the subfamily Hydrophiinae (Marine and Australo-Melanesian elapids) within the family Elapidae ("front-fanged" venomous snakes). *Naja haje* (common name: Egyptian cobra) is a "true" cobra species of the subfamily Elapinae (African, American, and Asian elapids) within the family Elapidae (Slowinski and Keogh 2000).

Cloning of the *Naja haje* Ligand Binding Domain and Construction of Chimeric/Mutated nAChRs

Materials and methods used in cloning the *Naja haje* ligand binding domain and constructing chimeric/mutated nAChRs is described in detail by Takacs (1997) and Takacs et al. (2001). In summary, total RNA was isolated using a CLONsep Total RNA Isolation Kit (Clontech, Palo Alto, CA). One gram of excised tissue was flash-frozen in liquid nitrogen, pulverized with a mortar and pestle under liquid nitrogen, then processed according to the manufacturer's instructions. The quality of the final RNA product was evaluated on an agarose gel. First-strand cDNA was reverse transcribed using oligo (dT)₁₈ primers by M-MLV reverse transcriptase with the 1st-STRAND cDNA synthesis kit (Clontech). First-strand cDNA was used as template for amplification of the ligand binding domain by PCR with primer pairs 5'-CACCT ATTTC CCCTT TGATG AGCA-3' and 5'-ATGAT GACGT TGACA ATGAA GTAGA GA-3'. The PCR product was used to construct a *Naja/Mus* chimeric α subunit, α N1. α N1 was used as the template for subsequent chimera and point mutations constructed by PCR mutagenesis with sets of primers described by Takacs et al. (1997). The presence of glycosylation was monitored by western blot analysis (Takacs et al. 2001).

Expression in *Xenopus* Oocytes

nAChR subunits α , mutated α , β , γ , and δ cDNAs, were linearized to completion and treated with 200 μ g/ml proteinase K in the presence of 0.5% SDS for 1 h at 50°C, then extracted for 1 min with 1 vol of phenol:chloroform:isoamyl alcohol (P:C:I; 25:24:1) followed by centrifugation at 15,000g for 5 min. The aqueous phase

was saved and the P:C:I extraction repeated two more times, followed by two chloroform:isoamyl alcohol (24:1) extractions under the above conditions. The aqueous phase was precipitated with 2 vol of ethanol in the presence of 0.2 vol NH₄-acetate, 100 mM EDTA at -80°C. The sample was centrifuged at 15,000g and 4°C for 30 min, and the pellet washed with 70% ethanol, then centrifuged at 15,000g and 4°C for 30 min. After discarding the supernatant, the pellet was vacuum-dried for 10 min and dissolved in 6–20 μ l H₂O. cRNA was transcribed in vitro by mMessage mMachine kit (Ambion, Austin, TX). In the 20- μ l total reaction volume 1.0–1.5 μ g linearized cDNA was mixed with SP6 RNA polymerase enzyme mix in the presence of ribonuclease inhibitor, a 5 mM concentration of each, ATP, CTP, and UTP, 1 mM GTP, and 4 mM Cap analogue in the transcription buffer and incubated at 37°C for 2 h. The reaction was terminated by 1 μ l RNase-free DNase I (2 U/ μ l) and incubation at 37°C for 15 min. After removal of the template DNA, 115 μ l H₂O, 15 μ l of 5M ammonium-acetate, and 100 mM EDTA were added, then extracted for 1 min with 1 vol P:C:I (25:24:1), followed by centrifugation at 15,000g for 2 min. The aqueous phase was saved and the P:C:I extraction repeated one more time, followed by two chloroform:isoamyl alcohol (24:1) extractions under the same conditions. The aqueous phase was precipitated with 1 vol of isopropyl alcohol or 3 vol of ethanol at -20°C for 30 min. The sample was centrifuged at 15,000g and 4°C for 30 min, and the pellet washed with 300 μ l of 70% ethanol, then centrifuged at 15,000g and 4°C for 30 min. After discarding the supernatant, the pellet was vacuum-dried for 10 min, and the cRNA was dissolved in 20–30 μ l H₂O and stored at -130°C. The quality and quantity of cRNA were evaluated and estimated by agarose gel electrophoresis. cRNAs were mixed at a molar ratio of 2 α : β : γ : δ , then 50 nl (200 pg/nl) was injected into the vegetal pole of the oocytes. Oocytes were incubated at 16°C and assayed between 24 and 72 h postinjection (Boyle and Kaczmarek 1991).

Acetylcholine-Induced Currents and Erabutoxin-a Dose-Response Recording

ACh-induced currents of the injected *Xenopus* oocytes were assayed by two-microelectrode voltage clamp at a holding potential of -40 mV, using electrodes filled with 3.3 M KCl at <1.5-M Ω resistance as described by Takacs et al. (2001). ACh (Sigma) was applied in the bath solution containing 115 mM NaCl, 2.5 mM KCl, 1.8 mM MgCl₂, 1 μ M atropine, 10 mM HEPES, pH 7.5, at a flow rate of 6 ml/min at room temperature. Oocytes were allowed to recover for 5–10 min after impalement with the electrodes before being exposed to 4 \times 10⁻⁵ M (ACh for 10 s, and the peak current amplitude (I_{max}) was recorded as the maximum change in holding current. At 5-min intervals, oocytes were exposed to various concentrations of ACh, ranging from 1 \times 10⁻⁷ to 2 \times 10⁻⁵ M for 10 s, and the peak current amplitude (I_{est}) was recorded as the maximum change in holding current. At the end of each protocol the application of 4 \times 10⁻⁵ M ACh for 10 s was repeated to ensure reproducibility. Representative normalized ACh dose-response relationships were calculated by fitting the peak currents to the Hill equation, $I = I_{max} / \{1 + (K_{app} / [ACh])^n\}$. Each data point displayed along the curves is the mean (I_{est}/I_{max}) \pm SE recorded from two to five individual oocytes.

ETX-a dose-response relationships were recorded 5–10 min after impalement with the electrodes, as oocytes were exposed twice (5 min apart) to 10⁻⁵ M ACh for 10 s and the peak current amplitudes averaged (I_{max}). Following these control recordings, the same oocytes were superfused with bath solution containing various concentrations of *Laticauda semifasciata* erabutoxin-a (ETX-a) as indicated in the figures for 10 min, then exposed again twice (5 min apart) to 10⁻⁵ M ACh for 10 s in the continuous presence of the ETX-a. The peak current amplitudes from at least two ACh exposures were averaged (I_{est}). I_{est}/I_{max} values were plotted as a

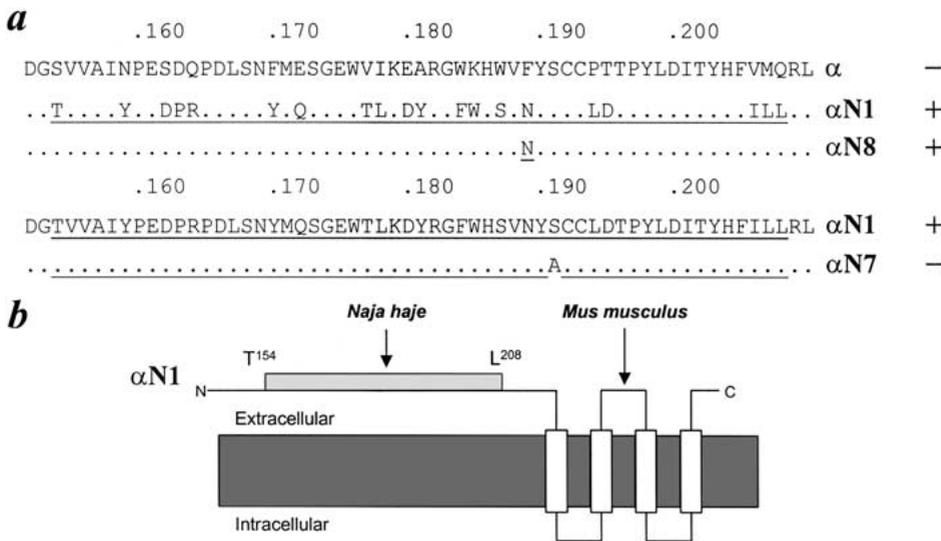


Fig. 1. Structure of the nAChR α subunits used in this study. **a** Ligand binding domain amino acid sequence of the chimeric and point mutated α subunits. α , wild-type *Mus musculus* α subunit; α N1, *M. musculus* chimeric α subunit encompassing the entire *N. haja* ligand binding domain; α N8, *M. musculus* α subunit carrying the *N. haja* glycosylation signal; α N7, *M. musculus* chimeric α subunit encompassing the entire *N. haja* ligand binding domain from which the glycosylation signal is removed. For clarity,

underlined residues are from *N. haja*; all others are from *M. musculus*. Dots indicate residues identical to the uppermost sequence in the panel, α or α N1. + or — indicates whether the consensus sequence for *N*-glycosylation is present (+) or absent (—). **b** Schematic representation of the chimeric α N1 subunit. The shaded segment T¹⁵⁴–L²⁰⁸ represents the region originating from the *N. haja*; the rest of the molecule is from the *M. musculus*.

function of ETX-a concentration. Representative normalized dose–response curves were calculated by fitting the peak currents to the Hill equation, $I = I_{\max} / \{1 + (K_{\text{app}} / [\text{ETX-a}])^n\}$. Each data point displayed for both ETX-a sensitive and ETX-a resistant nAChRs along the curves are the means of $(I_{\text{test}}/I_{\max}) \pm \text{SE}$ recorded from 2–13 individual oocytes.

Results

In order to test the hypothesis that the *N*-linked glycosylation present on the cobra, *Naja* spp., Elapidae, nAChR ligand binding domain interferes with the action of sea snake short-chain α -neurotoxin ETX-a, we constructed three chimeric/point mutated nAChR α subunits (Fig. 1). α N1 contained the full ligand binding domain of the Egyptian cobra (*Naja haja*, Elapidae) nAChR T¹⁵⁴–L²⁰⁸ flanked by the wild-type mouse (*M. musculus*) nAChR sequences (α). α N7 was the same as α N1, except that the S191A point mutation was introduced, which eliminates the consensus sequence for *N*-glycosylation that occurs naturally in the cobra, *Naja* spp., nAChR. α N8 was constructed by introducing the F189N point mutation to the wild-type mouse nAChR sequence, which creates the *N*-glycosylation signal present in *Naja* spp. These α subunits were coexpressed with the wild-type mouse β , γ , and δ subunits in *Xenopus* oocytes. All constructs were assayed for ACh-induced currents by two-microelectrode voltage clamp to ensure that the mutation does not alter normal receptor function. The peak current amplitudes, individual time courses, and dose–response characteristics of

ACh-induced responses were comparable to those of the wild-type mouse nAChR in all three constructs (Figs. 2 and 4). In addition, western blot analysis showed the expected mobility change according to the presence or lack of *N*-glycosylation consensus sequence in the α subunits.

Upon exposure to various concentrations of ETX-a (ranging from 6.9×10^{-10} to 6.9×10^{-7} M), the whole-cell currents induced by 1×10^{-5} M ACh were inhibited in the wild-type mouse α -nAChR (Fig. 2) in a concentration-dependent manner. The dose–response relationship of the inhibition by ETX-a of α -nAChR resulted in an IC_{50} of 3.7×10^{-9} M (Fig. 3). However, under the same conditions, α N1-nAChR, which contains the full *Naja* ligand binding domain exposed to 6.9×10^{-7} M ETX-a was only inhibited to $\sim 59\%$ of the maximal response, reflecting about a ~ 320 -fold shift of the IC_{50} (estimated IC_{50} , 1.2×10^{-6} M) (Figs. 2 and 3).

The inhibitions of α N7-nAChR and α N8-nAChR by ETX-a were intermediate between those of α -nAChR and α N1-nAChR (Fig. 4). The dose–response relationship of the inhibition by ETX-a in α N7-nAChR, containing the full *Naja* ligand binding domain from which the glycosylation was eliminated by a point mutation, resulted in an IC_{50} of 5.7×10^{-8} M. Under the same conditions, α N8-nAChR, carrying the *Naja* glycosylation in the full mouse background, yielded a more resistant channel to ETX-a, with an IC_{50} of 3.3×10^{-7} M (Fig. 5).

These results demonstrate that the *N*-linked glycosylation signal that is naturally present in the *Naja*

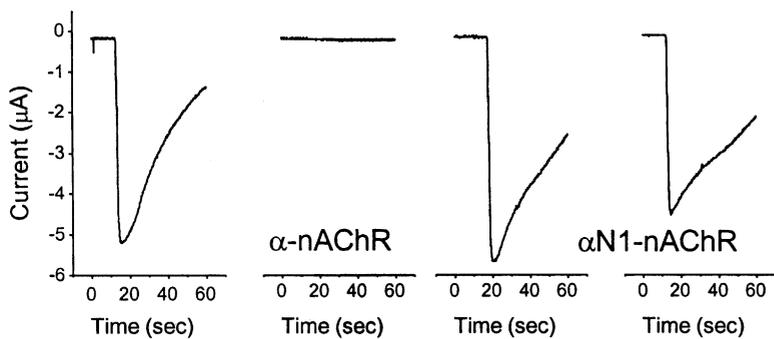


Fig. 2. ETX-a inhibition of ACh-induced whole-cell currents of α -nAChR and α N1-nAChR. α N1-nAChR is only partially inhibited by ETX-a. Representative whole-cell current records induced by the application of 10^{-5} M ACh for 10 s in a single oocyte injected with α -nAChR (two left traces) and α N1-nAChR (two right traces) cRNA. Current records in the absence of ETX-a (left traces of each pair) and after a 15-min exposure to 3.6×10^{-8} M ETX-a (right traces of each pair).

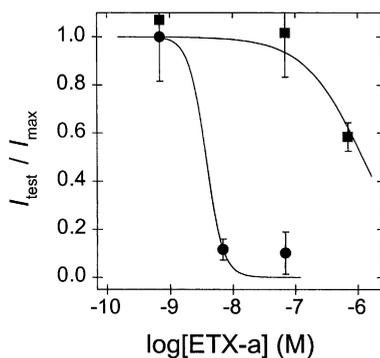


Fig. 3. ETX-a dose-response relationship of α -nAChR and α N1-nAChR. Normalized ETX-a dose-response relationship of (circles) α -nAChR and (squares) α N1-nAChR. Average peak current amplitudes plotted \pm SE. Refer to Materials and Methods for experimental details and curve fitting.

ligand binding domain confers protection against the inhibitory action of the short-chain α -neurotoxin ETX-a. If this glycosylation is selectively removed and the *Naja* ligand binding domain is exposed, the nAChR becomes sensitive to ETX-a, however, to a lesser extent than in the wild-type mouse.

Discussion

Natural Resistance to Short-Chain Snake α -Neurotoxin

Snakes and other venomous and poisonous organisms are resistant to components of conspecific venoms and poisons. Of the crude snake venom from the Elapidae family, α -neurotoxins considered to be among the major lethal components against prey and predator species, as well as humans. The present study demonstrates that the pharmacological action of short-chain snake α -neurotoxin on the nAChR is inhibited by the cobra, *Naja* spp., nAChR ligand binding domain T¹⁵⁴-L²⁰⁸. Selective elimination or introduction of the *N*-linked glycosylation signal that is naturally present in the *Naja* nAChR

at position 189 revealed that this glycosylation is a major factor for conferring resistance. When the *N*-glycosylation was removed by point mutation from the intact *Naja* ligand binding domain T¹⁵⁴-L²⁰⁸, the nAChR was blocked by the short-chain α -neurotoxin, although to a lesser extent than seen in the wild-type mouse nAChR. This difference could be explained by inherent species variations in α -neurotoxin sensitivity. Ligand binding domain residues, in addition to the *N*-glycosylation consensus sequence, could be involved in contributing resistance to α -neurotoxin.

Our earlier studies addressing the molecular mechanism of natural resistance to snake venom α -neurotoxins used α -BTX, a member of the long-chain α -neurotoxins. It has been concluded that α -BTX resistance is due to the glycosylation of N¹⁸⁹ naturally present in the Elapidae ligand binding domain (Takacs et al. 2001). This conclusion is also supported by the reduced binding affinity of α -BTX on the mouse nAChR carrying the homologous glycosylation signal (Kreienkamp et al. 1994; Keller et al. 1995). Here we showed that, similarly to α -BTX, the glycosylation of the Elapidae nAChR ligand binding domain at N¹⁸⁹ also inhibits the pharmacological action of ETX-a, albeit to a lesser extent. Compared to α -BTX, the quantitatively slightly lesser effect of the glycosylation on the activity of ETX-a could be explained by the difference in the pharmacological characteristics between short-chain and long-chain α -neurotoxins, which, in turn, reflects dissimilarities in their binding sites/mechanisms. Several lines of evidence, including binding kinetics to *Torpedo* membrane nAChRs (Chicheportiche et al. 1975), binding pattern to synthetic peptide fragments of various species, biotinylation experiments (Neumann et al. 1986; Ruan et al. 1991; Spura et al. 2000), and differential inhibition of short-chain and long-chain α -neurotoxin binding to nAChR by several conotoxins (Stiles, 1993), support this fact. In addition, the presence of invariant residues in short-chain α -neurotoxins that are not found in long-chain α -neuro-

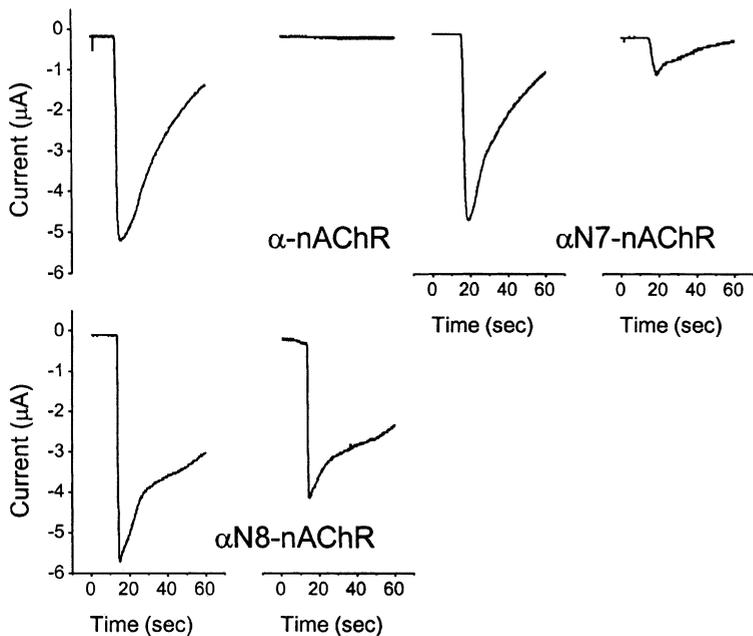


Fig. 4. ETX-a inhibition of ACh-induced whole-cell currents of α -nAChR, α N7-nAChR, and α N8-nAChR. α N7-nAChR is sensitive, and α N8-nAChR is resistant, to ETX-a. Representative whole-cell current records induced by the application of 10^{-5} M ACh for 10 s in a single oocyte injected with α -nAChR (two upper-left traces), α N7-nAChR (two upper-right traces), and α N8-nAChR (two lower-left traces) cRNA. Current records in the absence of ETX-a (left traces of each pair) and after a 15-min exposure to 3.6×10^{-8} M ETX-a (right traces of each pair).

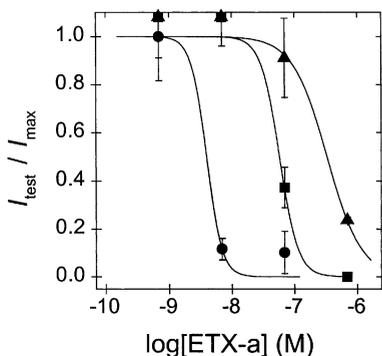


Fig. 5. ETX-a dose-response relationship of α -nAChR, α N7-nAChR, and α N8-nAChR. Normalized ETX-a dose-response relationship of (circles) α -nAChR, (squares) α N7-nAChR, and (triangles) α N8-nAChR. Average peak current amplitudes plotted \pm SE. Refer to Materials and Methods for experimental details and curve fitting.

Evolutionary Conservation of the Short-Chain Snake α -Neurotoxin Binding Site in Elapidae

For maximum effectiveness in predation and defensive strategy, snake venom neurotoxins must be targeted against evolutionary conserved molecular structures. The present results provide experimental evidence supporting this hypothesis. When the unique glycosylation was removed from the Elapidae ligand binding domain background, the nAChR become ETX-a sensitive. This susceptibility to ETX-a is explained by the conservation of ligand binding domain residues identified as required for binding other members of short-chain α -neurotoxins, W¹⁴⁹, V¹⁸⁸, Y¹⁹⁰, C¹⁹², C¹⁹³, P¹⁹⁷, Y¹⁹⁸, and D²⁰⁰ (Negrerie et al. 1991; Ackermann and Taylor 1997; Ackermann et al. 1998; Malany et al. 2000). We suggest that, contrary to earlier proposals (McLane et al. 1991; Ohana et al. 1991; Chaturvedi et al. 1992, 1993; Fuchs et al. 1993; Barchan et al. 1995; Kachalsky et al. 1995), these residues, among others located outside of the ligand binding domain, are targeted by short-chain and long-chain α -neurotoxins because they have a key physiological role, e.g., binding the natural transmitter, in the nAChRs of all Chordata. Therefore, due to the evolutionary pressure to conserve the ligand binding domain, Elapidae snakes could not afford structural alterations on the polypeptide backbone per se as an option to achieve resistance.

The fact that the selective elimination of glycosylation from the *Naja* ligand binding domain T¹⁵⁴-L²⁰⁸ does not yield a nAChR that is completely equal in ETX-a sensitivity to the mouse nAChR is expected and could be correlated with the well-documented

toxins (Endo and Tamiya 1991) and the lack of recognition of short-chain α -neurotoxins by monoclonal antibodies raised against long-chain α -neurotoxins where the epitope overlaps the nAChR binding site, and vice versa (Charpentier et al. 1990; Stiles et al. 1994), further indicate that there must be at least some differences in the mechanism of action on the nAChR for the two classes of snake α -neurotoxins. We interpret that the differences between the effects of glycosylation on α -BTX and ETX-a reflects intimate dissimilarities between the binding sites/mechanisms of the two classes of α -neurotoxins. The results of the current study have direct implications in terms of the evolution of snake neurotoxins and evolution of natural resistance.

culus (Boidae) and *Natrix tessellata* (Colubridae) at the homologous position to the two species of cobras, *Naja atra* and *N. haje*, sequenced to date (Neumann et al. 1989; Takacs et al. 2001). Boidae is universally regarded as more basal than the Elapidae or other members of the Colubroidea (contains all present-day venomous, including the “rear-fanged,” snake species) (Wilcox et al. 2002).

In agreement with and extending the molecular data, in Chordata, there is a ubiquitous occurrence of α -neurotoxin sensitivity ranging from Chondrichthyes to Mammalia. There are only two known exceptions, select taxa of the order Squamata, Reptilia, and the mongooses, genus *Herpestes*, Mammalia, whose diet includes, in part, cobras. Squamata contains all species of lizards and snakes. Of the lizards, the mostly basal *Anolis*, *Agama*, *Gekko*, *Lacerta*, and *Cordylus* are all sensitive to α -neurotoxins (Burden et al. 1975; Neumann et al. 1989). The first α -neurotoxin-resistant lizard species is an anguimorph lizard, *Ophisaurus ventralis* (Anguimorpha: Anguidae), which exhibits resistance to both short-chain and long-chain snake α -neurotoxins (Burden et al. 1975). Likewise, *Varanus exanthematicus* (Varanoidea: Varanidae), an other member within the Anguimorpha clade, exhibits resistance against cobra (*Naja* spp.) venom. Anguimorphs are a group of lizard families with numerous uniquely shared traits and several of Anguimorpha/limbless Scleroglossa characters (forked tongue, reduction or loss of the left lung, loss of external and middle ears, lidless eye protected by spectacles) are also found in snakes (Zug 1993). Snakes (suborder Serpentes) likely arose from Anguimorphs (Forstner et al. 1995). Of snakes, all species studied to date except *Epicrates* (Burden et al. 1975) are α -neurotoxin resistant. *Epicrates*, however, still shows resistance to short-chain α -neurotoxin, but not to α -BTX (Burden et al. 1975). Specifically, α -neurotoxin-resistant snake species identified to date include the Colubridae genera *Rhabdophis* (Endo and Tamiya 1991), *Elaphe* (Burden et al. 1975; Liu et al. 1990; Endo and Tamiya 1991), *Thamnophis* (Burden et al. 1975), and *Natrix* (Neumann et al. 1989); the Elapidae genera *Naja* (Liu et al. 1990; Z. Takacs, personal observation), *Bungarus* (Yan-Qin and Jing-Yu 1998), and *Walterinnesia* (Ovadia and Kochva 1977); and the Viperidae genera *Agkistrodon* (Liu et al. 1990; Endo and Tamiya 1991) and *Trimeresurus* (Endo and Tamiya 1991). Of sea snakes, *Pelamis* also exhibits a high resistance to conspecific venom (Pickwell 1994). It remains to be determined where exactly the glycosylation of N¹⁸⁹ on the muscle nAChR α -subunit appeared and what its initial selective advantage was.

Model of α -Neurotoxin Resistance in Snakes

Based on the present findings, our earlier studies on long-chain α -neurotoxin (Takacs et al. 2001), and the

available literature, the following working model is proposed to explain the α -neurotoxin resistance in Elapidae snakes (Fig. 6). This model rejects the previous proposal that the absence of aromatic amino acid residues at positions 187 and 189 would mediate resistance against α -neurotoxins of the nAChR (Kachalsky et al. 1995). In addition, the lack of a proline residue at position 194 in Elapidae as a further explanation for resistance to α -neurotoxins (Chaturvedi et al. 1992, 1993; Kachalsky et al. 1995) is also rejected. The proposal that the Elapidae ligand binding domain is α -neurotoxin resistant due to amino acid changes per se at individual positions in the linear polypeptide backbone (McLane et al. 1991; Ohana et al. 1991; Chaturvedi et al. 1992, 1993; Fuchs et al. 1993; Barchan et al. 1995; Kachalsky et al. 1995) is rejected as well. The model is supported by, and extends, the description of the involvement of glycosylation in reducing α -BTX affinity (Kreienkamp et al. 1994; Keller et al. 1995). According to the model, the part of the binding site for short-chain and long-chain snake α -neurotoxins that is carried by the T¹⁵⁴–L²⁰⁸ segment in α -neurotoxin-sensitive species is also conserved in the Elapidae nAChR α subunit per se. Namely, these are residues W¹⁴⁹, V¹⁸⁸, Y¹⁹⁰, C¹⁹², C¹⁹³, P¹⁹⁷, Y¹⁹⁸, and D²⁰⁰ for short chain α -neurotoxins (Negrerie et al. 1991; Ackermann and Taylor 1997; Ackermann et al. 1998; Malany et al. 2000) and H¹⁸⁶, V¹⁸⁸, C¹⁹², C¹⁹³, D¹⁹⁵, P¹⁹⁷, Y¹⁹⁸, and D²⁰⁰ for long-chain α -neurotoxins (references in Takacs 1997). The primary amino acid sequence backbone in the T¹⁵⁴–L²⁰⁸ segment of Elapidae is capable of forming a binding site for α -neurotoxins. This binding site leads to the inhibition of nAChR function to the same or a slightly lesser extent as in α -neurotoxin-sensitive species. Consequently, amino acid residues per se in the T¹⁵⁴–L²⁰⁸ segment of Elapidae may cause subtle changes in α -neurotoxin sensitivity that reflect species differences, but they do not confer α -neurotoxin resistance to the nAChR. Conservation of the α -neurotoxin binding site in T¹⁵⁴–L²⁰⁸ segment in Elapidae reflects, and is part of, an overall conservation of nAChR structure that is indispensable for the physiological function. α -Neurotoxin resistance is mediated, however, by the presence of an oligosaccharide attachment to residue N¹⁸⁹ that is part of an N-glycosylation consensus sequence, N¹⁸⁹–Y¹⁹⁰–S¹⁹¹. Based on the signal sequence (Wagh and Bahl 1981; Kornfeld and Kornfeld 1976), the core of this oligosaccharide is Man α 1 \rightarrow 3-(Man α 1 \rightarrow 6) Man β 1 \rightarrow 4GlcNAc β 1 \rightarrow 4GlcNAc-N¹⁸⁹; the outer branches are unknown. N¹⁸⁹ is positioned in the vicinity of a cluster of amino acid residues that potentially serve, directly or indirectly, as the primary determinants for α -neurotoxin binding/activity. The intimate molecular mechanism of the glycosylation itself prevents the inhibition of the nAChR physiological function by

altering the conformation and stability of the α -neurotoxin–nAChR bound complex, and not simply by restricting the diffusional access (Keller et al. 1995). Acting synergistically with glycosylation, L¹⁹⁴ may provide the rotational freedom of the polypeptide backbone that enables the oligosaccharide to alter the conformation of the nAChR's α -neurotoxin binding domain (Keller et al. 1995). The glycosylation at N¹⁸⁹ does not interfere with the physiological binding of the natural ligand, ACh, or with the ACh-induced conformational changes leading to channel opening and subsequent states of the nAChR (Takacs et al. 2001). Residues identified to form, directly or indirectly, the ACh binding sites, Y¹⁹⁰, C¹⁹²–C¹⁹³, and Y¹⁹⁸, are conserved in Elapidae and fulfill the same function as in other taxa. Binding sites for nAChR-specific ligands other than snake α -neurotoxins, such as carbamylcholine, lophotoxin (Y¹⁹⁰), α -conotoxin M1 (Kreienkamp et al. 1994), and *d*-tubocurarine (Y¹⁹⁰), are conserved in Elapidae. This conservation, again, reflects and is part of an overall conservation of nAChR structure that is indispensable for the physiological function. The data presented here cannot exclude the possibility that in the Elapidae, nAChR structural elements outside of the α subunit T¹⁵⁴–L²⁰⁸ segment also contribute to the resistance. In terms of evolution of resistance, based on sequence information, we suggest that glycosylation of N¹⁸⁹ appeared earlier than Elapidae snakes and facilitated the evolution of snake venom α -neurotoxins. We do not assume that this description is necessarily final: rather it is a working model based on the current knowledge and is subject to changes according to future experimentation.

Conspecific Neurotoxin Resistance in Other Animals

While the present study was carried out on snakes, we suggest that many other poisonous and venomous animals evolved a similar strategy of escaping the effects of their own neurotoxins, namely, an intimate alteration of the structure of the toxin binding site while preserving the physiological function of the receptor. This newly described mean of resistance differs from the natural resistance mediated by humoral factors present in the blood plasma of vipers (Viperidae), the only other mechanism of protection described (Straight et al. 1976). Nerve and/or muscle preparations free of hemolymph or plasma from the scorpion, *Androctonus australis* (Legros et al. 1998), various species of newts, Amphibia: Urodela (Kaneko et al. 1997), and the poison-dart frog, *Phylllobates terribilis* (Daly et al. 1980) are all insensitive to conspecific neurotoxins. Furthermore, there are many other poisonous animal species where the neurotoxin is widely distributed at high concentrations in various tissues of the body, such as puffer fish and other species of

dendrobatid frogs (Yoshida 1994). These examples indicate that in conspecifics, the toxin's target is most likely structurally modified at the molecular level.

Conclusions

In summary, the presence of a unique glycosylation in the cobra, *Naja* spp., nAChR ligand binding domain at position 189 inhibits the effect of the short-chain snake α -neurotoxin, ETX-a. Removal of the glycosylation that is flanked by the native Elapidae ligand binding domain sequence will convert the nAChR to be ETX-a sensitive, nearly to the value observed in mouse nAChR. In conclusion, these results indicate that (i) short-chain and long-chain α -neurotoxins differ in their intimate mechanism of action/species specificity, (ii) glycosylation is a primary or a major cause of resistance against conspecific snake short-chain α -neurotoxins, and (iii) snake venom α -neurotoxins are targeted against evolutionary conserved molecular structures. In addition, the presence of glycosylation consensus sequence and a phylogenetic analysis of snake venom α -neurotoxin sensitivity suggest that α -neurotoxin resistance appeared first, which permitted the evolution of snake venom α -neurotoxins. The description of the Elapidae ligand binding domain presented here should serve as a working model to explore further the evolutionary and pharmacological basis of natural resistance to snake venom α -neurotoxins.

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