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Inter-residue coupling contributes to high-affinity subtype-selective binding of α -bungarotoxin to nicotinic receptors

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Abstract

The crystal structure of a pentameric $\alpha 7$ ligand-binding domain chimaera with bound α -btx (α -bungarotoxin) showed that of the five conserved aromatic residues in $\alpha 7$, only Tyr¹⁸⁴ in loop C of the ligand-binding site was required for high-affinity binding. To determine whether the contribution of Tyr¹⁸⁴ depends on local residues, we generated mutations in an $\alpha 7/5HT_3A$ (5-hydroxytryptamine type 3A) receptor chimaera, individually and in pairs, and measured ¹²⁵I-labelled α -btx binding. The results show that mutations of individual residues near Tyr¹⁸⁴ do not affect α -btx affinity, but pairwise mutations decrease affinity in an energetically coupled manner. Kinetic measurements show that the affinity decreases arise through increases in the α -btx dissociation rate with little change in the association rate. Replacing loop C in $\alpha 7$ with loop C from the α -btx-insensitive $\alpha 2$ or $\alpha 3$ subunits abolishes high-affinity α -btx binding, but preserves acetylcholine-elicited single channel currents. However, in both the $\alpha 2$ and $\alpha 3$ construct, mutating either residue that flanks Tyr¹⁸⁴ to its $\alpha 7$ counterpart restores high-affinity α -btx binding. Analogously, in $\alpha 7$, mutating both residues that flank Tyr¹⁸⁴ to the $\alpha 2$ or $\alpha 3$ counterparts abolishes high-affinity α -btx binding. Thus interaction between Tyr¹⁸⁴ and local residues contributes to high-affinity subtype-selective α -btx binding.

Keywords

crystal structure; inter-residue coupling; molecular recognition; neurotoxin; nicotinic acetylcholine receptor

INTRODUCTION

Long chain α -neurotoxins, such as α -btx (α -bungarotoxin) and α -ctx (α -cobratoxin), are renowned for their high-affinity slowly reversible binding to nicotinic AChRs (acetylcholine

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AUTHOR CONTRIBUTION

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receptors) [1,2]. The physiologically relevant AChR is located within the post-synaptic membrane of voluntary muscle, and contains two acetylcholine-binding sites coupled to an intrinsic ion channel. Binding of either α -btx or α -ctx to the AChR prevents binding of nerve-released acetylcholine, resulting in paralysis. Onset of paralysis is slow, owing to a toxin-association rate several orders of magnitude lower than that of a diffusion-limited reaction, but recovery is prolonged due to a very slow rate of dissociation [3,4]. The twin properties of high affinity and low dissociation have made α -neurotoxins valuable labels for tracking and quantifying AChRs [5]. Furthermore, because binding of α -neurotoxins and small-molecule agonists or antagonists is mutually exclusive, radiolabelled α -neurotoxins have been employed to determine fractional occupancy by unlabelled ligands [4,6,7].

Within the pentameric AChR, each ligand-binding site is formed at the interface between a principal and a complementary subunit [8–10]. Together, the principal and complementary subunits contribute seven binding-site loops, each containing several consecutive residues from a different section of the aminoacid sequences of the subunits. The principal subunit contributes loops A–C, whereas the complementary subunit contributes loops D–G. Naturally occurring sequence variants within loop C are present in animals resistant to α -neurotoxins [11,12], whereas short peptides derived from loop C bind to α -neurotoxins [13–15]. Furthermore, mutational analyses showed that loop C was the most important of the seven binding site loops in conferring high affinity for α -btx [16,17], and replacing loop C from the muscle AChR for the analogous structure in the pentameric bacterial channel GLIC (*Gloeobacter violaceus* pentameric ligand-gated ion channel) yielded high-affinity α -btx binding [18]. The key importance of loop C was confirmed by crystal structures of α -neurotoxins bound to receptor or receptor-like targets where loop C lodged between two of the three fingers of the α -neurotoxin [19–21]. The complex between α -btx and an $\alpha 7$ /AChBP (acetylcholine-binding protein) chimera revealed a large area of inter-protein contact, but only a single focal interaction was essential for high-affinity binding [21]. The stabilizing residues included a conserved tyrosine residue within loop C of $\alpha 7$ and conserved arginine and phenylalanine residues of α -btx. Remarkably, none of the four remaining canonical aromatic residues of the $\alpha 7$ ligand-binding site were essential for high-affinity binding.

In addition to binding with high affinity, long-chain α -neurotoxins are noted for their target selectivity [22–24]. The AChR family is diverse, comprising ten subtypes of α -subunits, four types of β -subunits, and γ -, ϵ - and δ -subunits. Some subunits form homopentamers, such as $\alpha 7$, or more commonly, heteropentamers, of which there are many subunit combinations. α -Neurotoxins bind tightly to homo- or hetero-pentameric AChRs that contain $\alpha 1$, $\alpha 7$, $\alpha 8$, $\alpha 9$ or $\alpha 10$ subunits, but they do not bind to hetero-pentamers containing $\alpha 2$, $\alpha 3$ or $\alpha 4$ subunits. This target selectivity occurs despite the fact that all AChR α -subunits contain a signature loop C in which five of 12 residues are conserved (Figure 1). Insensitivity of $\alpha 2$ – $\alpha 4$ receptors to α -neurotoxins may potentially arise from differences in the variable residues of loop C, analogous to receptors in α -neurotoxin-resistant animals.

To identify structural elements that contribute to α -neurotoxin high affinity and target selectivity, we designed mutations in an $\alpha 7$ AChR based on the crystal structure of the complex between α -btx and a pentameric $\alpha 7$ ligand-binding domain chimera [21]. In particular, we sought to determine whether the contribution of the essential tyrosine residue in loop C of $\alpha 7$ depends on nearby residues defined in the structure. The results show that, in contributing to high affinity and subtype-selective α -btx binding, the essential tyrosine residue depends on nearby conserved and non-conserved residues.

EXPERIMENTAL

Expression of AChRs in HEK (human embryonic kidney)-293 cells

A cDNA encoding the high-conductance form of the $\alpha 7/5\text{HT}_{3\text{A}}$ (5-hydroxytryptamine type 3A) chimaeric receptor, subcloned in the expression vector pRBG4 [25], was transfected into HEK-293 cells using the calcium phosphate precipitation method as described previously [26]. Mutations were generated using the QuikChange[®] site-directed mutagenesis kit (Stratagene) and confirmed by sequencing the entire coding region.

Concentration dependence of ^{125}I -labelled α -btx binding

Transfected cells were harvested by gentle agitation in PBS, centrifuged at 1000 *g* for 1 min and resuspended in potassium Ringer's solution (140 mM KCl, 5.4 mM NaCl, 1.8 mM CaCl_2 , 1.7 mM MgCl_2 , 25 mM Hepes, pH 7.4). Cell suspensions were incubated with specified concentrations of ^{125}I -labelled α -btx (PerkinElmer) for 4 h at 21 °C and then toxin–receptor complexes were separated from free toxin using a Brandel M-48T cell harvester. Non-specific binding could not be determined by incubation in the presence of a competitive ligand, because many of the mutant AChRs impaired binding of small-molecule agonists and antagonists. Also, unlabelled α -btx is not suitable because it can displace the chemically similar ^{125}I -labelled α -btx from non-specific sites, and its affinity is markedly reduced by several mutations. Thus non-specific binding was determined by applying identical procedures to cells transfected with an equal amount of the pRBG4 vector containing the muscle AChR δ -subunit cDNA, which induces protein expression but does not form AChRs. At a saturating concentration of 30 nM ^{125}I -labelled α -btx, non-specific binding averaged 3.4 % of that obtained for the control $\alpha 7/5\text{HT}_{3\text{A}}$ receptor [21]. After subtracting non-specific binding, the amount of bound ^{125}I -labelled α -btx was expressed as a percentage of the maximum binding obtained for the control non-mutant $\alpha 7/5\text{HT}_{3\text{A}}$ receptor, and the Hill equation was fitted to percentage occupancy as a function of ^{125}I -labelled α -btx concentration using GraphPad Prism 5 software.

Kinetics of ^{125}I -labelled α -btx association

To measure the time course of α -btx association, a cell suspension prepared as described above was mixed with a specified concentration of ^{125}I -labelled α -btx at 21 °C, and aliquots of the suspension were rapidly filtered through type A/E glass fibre filters (Gelman Sciences) at specified times. To determine non-specific binding, identical procedures were applied to cells transfected with the pRBG4 vector containing the muscle AChR δ -subunit cDNA. For each type of AChR, separate binding time courses were determined for one to three concentrations of ^{125}I -labelled α -btx and analysed with Dynafit software [27].

Kinetics of ^{125}I -labelled α -btx dissociation

To measure the time course of α -btx dissociation, a cell suspension, prepared as described above, was mixed with a saturating concentration of ^{125}I -labelled α -btx (150–300 nM) and incubated for 1 h at 21 °C. The suspension was then centrifuged at 1000 *g* for 1 min, the supernatant was removed, and the cell pellet was resuspended in 30 ml of potassium Ringer's solution. Aliquots of the suspension were then rapidly filtered through type A/E glass fibre filters (Gelman Sciences) at specified times. To determine non-specific binding, identical procedures were applied to cells transfected with the pRBG4 vector containing the muscle AChR δ -subunit cDNA. After subtracting non-specific binding, a single exponential decay plus a variable plateau was fitted to the data using GraphPad Prism 5 software.

Patch-clamp recordings

Single-channel recordings were obtained in the cell-attached patch configuration [28] at a membrane potential of -70 mV at 21°C , essentially as described before [29,30]. The bath solution contained 142 mM KCl, 5.4 mM NaCl, 0.2 mM CaCl_2 and 10 mM Hepes, pH 7.4, whereas the pipette solution contained 80 mM potassium fluoride, 40 mM potassium aspartate, 2 mM MgCl_2 , 1 mM EGTA and 10 mM Hepes, pH 7.4. Patch pipettes were pulled from 7052 capillary tubes (King Precision Glass) and coated with Sylgard (Dow Corning). Single-channel currents were recorded using an Axopatch 200 B patch-clamp amplifier (Molecular Devices), digitized at $5\ \mu\text{s}$ intervals with the PCI-6111E interface (National Instruments), and detected by the half amplitude threshold criterion using the program TAC 4.0.10 (Bruxton Corporation) at a final bandwidth of 10 kHz [29,30]. Open-time histograms were plotted using a logarithmic abscissa and a square root ordinate [31] and fitted to the sum of exponentials by maximum likelihood using the program TACFit (Bruxton).

RESULTS

The complex between α -btx and $\alpha 7$ /AChBP

Mutational analyses were based on the crystal structure of α -btx bound to a pentameric $\alpha 7$ ligand-binding domain chimaera in which all residues within loop C and those that line the aromatic-rich binding cavity originate from the native human $\alpha 7$ receptor [21,32]. Despite a large area of contact in which the two binding partners exhibit shape and chemical complementarity, only a single aromatic residue, Tyr¹⁸⁴ in loop C of $\alpha 7$, is required for high-affinity binding of α -btx [21]. Located deep within the binding cleft, Tyr¹⁸⁴ interacts edge-to-face with Arg³⁶ and Phe³² of α -btx that form a π -cation stack at the tip of toxin finger II (Figure 2). Furthermore, Phe¹⁸³ of $\alpha 7$ inserts into a hydrophobic pocket between toxin fingers I and II, whereas Tyr¹⁹¹ from the C-terminus of loop C establishes an aromatic-cation interaction with Arg³⁶. To determine whether the contribution of Tyr¹⁸⁴ to high-affinity α -btx binding depends on local residues, we combined mutations of Tyr¹⁸⁴ with mutations of either Phe¹⁸³ or Tyr¹⁹¹.

Mutational analyses of single and pairwise mutations

To assess the contributions of candidate residues to α -btx binding, we generated mutations in an $\alpha 7$ /5HT₃ receptor chimaera that contains human $\alpha 7$ sequence from the N-terminus to the start of the first transmembrane domain, followed by 5HT₃ sequence to the C-terminus. This chimaeric AChR expresses robust quantities of cell-surface receptors in HEK-293 cells and exhibits single-channel currents in response to agonists [26,30]. For consistency with the $\alpha 7$ /AChBP crystal structure, residue positions in $\alpha 7$ /5HT₃ are numbered according to those in the crystal structure rather than native $\alpha 7$. To determine how the mutations affect α -btx binding, intact cells expressing receptors were incubated with specified concentrations of ¹²⁵I-labelled α -btx, and then bound complexes were separated from the free toxin using a cell harvester. Non-specific binding was determined by applying identical procedures to cells transfected with equal amounts of the pRBG4 vector containing the muscle AChR δ -subunit cDNA as described in the Experimental section. For the non-mutant $\alpha 7$ /5HT₃ receptor, specific binding of α -btx is saturable and exhibits an apparent K_d of approximately 8 nM and a Hill coefficient approaching two (Figure 3 and Table 1). Both parameters probably overestimate the true values because the incubation time of 4 h may not have been sufficient to reach equilibrium. Nevertheless, the apparent K_d is only 2-fold greater than that determined from the measurements of α -btx association kinetics described below.

To evaluate the contributions of aromatic residues at the ligand-binding site, we chose threonine as the replacement residue because it removes the aromatic ring, but retains a hydroxyl group. However, the Y184T mutation eliminates high-affinity α -btx binding [21],

so instead Tyr¹⁸⁴ was replaced with phenylalanine. The single residue mutations Y184F and Y191T do not affect the apparent K_d for α -btx (Figure 3a and Table 1). However, when both mutations are present in the same receptor, the apparent K_d increases approximately 6-fold. Analogously, the single residue mutations Y184F and F183T do not affect the apparent K_d (Figure 3b), but the pairwise mutations again increase the K_d . Mutant cycle analyses [33] applied to the pairs Y184F/Y191T and Y184F/F183T reveal coupling free energies of -1.0 and -0.8 kcal/mole ($1 \text{ cal} \approx 4.184 \text{ J}$) respectively (Table 1). Thus the contribution of Tyr¹⁸⁴ to high-affinity α -btx binding depends on the two proximal residues Tyr¹⁹¹ and Phe¹⁸³.

Because Tyr¹⁹¹ and Phe¹⁸³ are energetically coupled with Tyr¹⁸⁴, we asked whether the two residues are coupled with each other. Neither Y191T nor F183T affect α -btx affinity, as described above, but the pairwise mutation produces the largest decrease in affinity observed so far (Figure 3c). Mutant cycle analysis yields a large coupling free energy of -1.7 kcal/mole (Table 1). Thus, in view of the crystal structure of the complex (Figure 2), the observation of strong inter-residue energetic coupling suggests that Phe¹⁸³ and Tyr¹⁹¹ are essential to the proper alignment of Tyr¹⁸⁴ with the π -cation stack formed by Arg³⁶ and Phe³² of α -btx.

Within the principal face of the ligand-binding site, two more conserved aromatic residues, Trp¹⁴⁵ and Tyr⁹¹, interact with Arg³⁶ of α -btx; the guanidinium group of Arg³⁶ hydrogen bonds with the main chain carbonyl of Trp¹⁴⁵ and the aromatic hydroxyl of Tyr⁹¹ (Figure 2). The individual mutations W145T and Y91T do not affect α -btx affinity, neither do any of the four pairwise combinations with Y191T and F183T (Figure 3d and Table 1). Similarly, combining either W145T or Y91T with Y184F does not affect α -btx affinity (Table 1). Thus, although Trp¹⁴⁵ and Tyr⁹¹ contact the key Arg³⁶ of α -btx, neither of these canonical residues contributes to affinity alone or in combination with Tyr¹⁸⁴, Tyr¹⁹¹ or Phe¹⁸³.

A fifth conserved aromatic residue, Trp⁵³ from the complementary face of the ligand-binding site, contacts Phe³² of α -btx (Figure 2). The mutation W53T modestly reduces α -btx affinity, but the combination of W53T and F183T decreases affinity more than either mutation alone, yielding a coupling free energy of -0.85 kcal/mole. Also, the combination of W53T and Y184F yields a coupling free energy of -0.56 kcal/mole. On the other hand, Trp⁵³ shows weak or no coupling to Tyr¹⁹¹, Tyr⁹¹ or Trp¹⁴⁵ (Table 1). Thus Trp⁵³ couples energetically with Tyr¹⁸⁴ and Phe¹⁸³, perhaps through the intervening Phe³² of α -btx. Notably, in the $\alpha 7$ /AChBP crystal structure, Phe¹⁸³, Tyr¹⁸⁴, Trp⁵³ and Phe³² span a distance of $24 \text{ \AA}$ ($1 \text{ \AA} = 0.1 \text{ nm}$) between the principal and complementary subunits, and all four residues are co-linear.

Kinetics of ¹²⁵I-labelled α -btx association

To determine how interactions among residues Tyr¹⁸⁴, Tyr¹⁹¹ and Phe¹⁸³ affect the rate constants underlying binding of α -btx, we measured time courses of radiolabelled α -btx binding to $\alpha 7$ /5HT₃ receptors without and with pairwise mutations. For the control and pairwise mutant receptors, time courses of α -btx binding were determined in the presence of two or three concentrations of α -btx, and the program Dynafit [27] was used to fit a reversible bimolecular binding mechanism simultaneously to the global set of time courses for each mutant. In this mechanism, the toxin (T) binds to the receptor (R) to form a reversible toxin–receptor complex (TR), with an association rate constant k_{+1} and a dissociation rate constant k_{-1} .

For the control $\alpha 7/5HT_3$ receptor, simultaneous fitting describes the time courses for three concentrations of α -btx (Figure 4), and yields estimates of the association and dissociation rate constants (Table 2). The ratio of dissociation to association rate constants yields a binding dissociation constant of 4.8 nM, which is 2-fold smaller than that obtained from steady-state binding measurements (Figure 3 and Table 1). Furthermore, the association rate constant is approximately three orders of magnitude lower than that of a diffusion limited reaction, similar to previous determinations [3,4], whereas the dissociation rate constant yields a time constant of bound α -btx of approximately 33 min.

To measure the α -btx dissociation rate constant directly, cells expressing the $\alpha 7/5HT_3$ receptor were incubated with a saturating concentration of radiolabelled α -btx, unbound toxin was removed, and bound toxin was measured as a function of time. The dissociation time course shows an exponential decay, with a time constant of 48 min, which reaches a stable plateau of 78 % of the initial toxin–receptor complex (Figure 5a and Table 2). The significance of the stable binding plateau is uncertain, but a possible interpretation is that the toxin–receptor complex is initially reversible, but it subsequently converts to a very stable complex. Despite the presence of a stable plateau, the directly measured decay rate constant is similar to the dissociation rate constant measured from fitting the reversible bimolecular association mechanism to the time course of toxin association (Table 2). A stable plateau was not evident from the kinetics of α -btx association, presumably because the association time course is measured over 25 min, whereas the stable complex is evident after approximately 60 min.

For receptors with pairwise mutations of coupled residues, the time courses of α -btx association are well described by the reversible bi-molecular association mechanism (Figure 4). The fitted association rate constant is unchanged from control for the pairs Y184F/Y191T and Y184F/F183T, but is decreased almost 2-fold for the pair Y191T/F183T (Table 2). By contrast with the association rate constants, the dissociation rate constants increase markedly compared with control for all three pairwise mutations (Table 2). Direct measurements of radiolabelled α -btx dissociation again show an exponential decay followed by a stable plateau (Figure 5b), but for the pairwise mutants, the plateau is much smaller than that observed for the control or any of the single residue mutations (Figure 5a). Because the pairwise mutations increase the rate of dissociation, the smaller plateau suggests that its magnitude is determined by the stability of the initial toxin–receptor complex. Moreover, the directly measured dissociation rate constants are similar to those determined from fitting the reversible bi-molecular association mechanism to the time courses of toxin association (Table 2). Thus, for these three residue pairs, energetic coupling arises primarily from increases in the rate of α -btx dissociation from the initial toxin–receptor complex.

For the three pairwise mutants, the ratio of dissociation to association rate constants yields kinetically determined dissociation constants that are similar to those determined from measurements of steady-state binding of radiolabelled α -btx (Table 2). Mutant cycle analyses, based on the kinetically determined dissociation constants, yield significant inter-residue coupling free energies (Table 2). These were similar to those determined from steady-state binding measurements (Table 1), and the kinetic and steady-state measurements exhibit the same rank order of coupling free energies for the three sets of pairwise mutants. Thus for the pairs Y184F/Y191T, Y184F/F183T and Y191T/F183T, the results from kinetic measurements agree with the results from steady-state measurements.

Contributions of inter-residue coupling with α -btx selectivity for nicotinic receptor subtypes

The results so far show that three local residues within loop C of $\alpha 7$ contribute interdependently to high-affinity α -btx binding. However, neuronal AChRs containing $\alpha 2$ –

$\alpha 4$ subunits are insensitive to α -btx [22,23], and residues in loop C of these subunits differ from those in α -subunits that confer sensitivity to α -btx (Figure 1). To determine whether differences in loop C contribute to subtype selectivity for α -btx, we replaced loop C in the $\alpha 7/5HT_3$ receptor with loop C from either the $\alpha 2$ or $\alpha 3$ subunits, and measured steady-state binding of radiolabelled α -btx. Replacing loop C from either $\alpha 2$ or $\alpha 3$ abolishes high-affinity α -btx binding (Figures 6a and 6b); a small amount of specific binding is observed at α -btx concentrations greater than approximately 30 nM, suggesting that affinity is markedly reduced. Remarkably, in both the $\alpha 2$ and $\alpha 3$ constructs, replacing either of the residues that flank Tyr¹⁸⁴ with the equivalent residue from $\alpha 7$ restores high-affinity α -btx binding (Figures 6a and 6b). Because the single mutants show high-affinity binding, and the double mutants show immeasurably low-affinity binding, the two flanking residues in $\alpha 2$ and $\alpha 3$ are interdependent in contributing to high-affinity α -btx binding. However, the inter-residue coupling free energy, although undoubtedly large, cannot be quantified.

Analogously, replacing loop C from the muscle $\alpha 1$ subunit into the $\alpha 7/5HT_3$ receptor yields high-affinity α -btx binding, but mutating the two residues that flank Tyr¹⁸⁴ to their $\alpha 3$ counterparts abolishes high-affinity binding (Table 3). In loop C of $\alpha 1$, the C-terminal half diverges from that in $\alpha 3$, but substituting the C-terminal half of loop C of $\alpha 3$ maintains high-affinity binding (Table 3). Thus sequence differences in the C-terminal half of loop C do not contribute to the subtype selectivity of α -btx.

Finally, in $\alpha 7$, replacing the two residues that flank Tyr¹⁸⁴ to their $\alpha 3$ counterparts abolishes high-affinity α -btx binding, but replacing just one of the flanking residues maintains high-affinity binding (Table 3). Thus, within loop C of the $\alpha 2$, $\alpha 3$, $\alpha 1$ and $\alpha 7$ subunits, Tyr¹⁸⁴ and its two flanking residues are strongly interdependent in contributing to subtype-selective high-affinity α -btx binding.

A departure from the trend described above is observed when loop C from the $\alpha 4$ subunit is substituted into the $\alpha 7/5HT_3$ receptor. Steady-state binding measurements reveal that α -btx binds with high affinity to the $\alpha 4$ construct (Table 3). Notably, loop C of $\alpha 4$ has a glutamate residue at position 185, like $\alpha 7$, whereas $\alpha 2$ and $\alpha 3$ have aspartate and asparagine respectively (Figure 1). However, replacing Glu¹⁸⁵ with asparagine within loop C of the $\alpha 4$ construct abolishes high-affinity α -btx binding, analogous to the results for the $\alpha 2$ and $\alpha 3$ loop C replacements (Table 3). Thus α -btx insensitivity of $\alpha 4$ -containing nicotinic receptors does not arise solely from residue differences within loop C, suggesting other residues in $\alpha 4$ or the complementary β -subunit contribute. Nevertheless, the findings from substituting loop C of $\alpha 4$ into $\alpha 7$ confirm the importance of the residues flanking Tyr¹⁸⁴ in contributing to subtype-selective α -btx binding.

Steady-state binding of α -btx results from the combination of association and dissociation rate constants and a plateau of stable binding. Because steady-state measurements may not sufficiently sense changes in reaction steps underlying α -btx binding, we directly measured time courses of α -btx dissociation from $\alpha 7/5HT_3$ receptors with various loop C replacements. The construct with loop C from $\alpha 4$ shows accelerated dissociation of α -btx and a much lower plateau of binding compared with the control $\alpha 7/5HT_3$ receptor (Table 3). Similarly, constructs with the glutamate residue substituted at position 185 of either $\alpha 2$ or $\alpha 3$, while retaining the lysine residue at position 183, show accelerated dissociation of α -btx and a lower plateau (Table 3). On the other hand, in both the $\alpha 2$ and $\alpha 3$ constructs, substituting the phenylalanine residue at position 183 and the glutamate residue at position 185 decreases the rate of α -btx dissociation and increases the plateau, mimicking the control $\alpha 7/5HT_3$ receptor (Table 3). These findings confirm that residues flanking the essential Tyr¹⁸⁴ contribute both to the stability of the initial toxin–receptor complex and the magnitude of the stable plateau.

Four of the constructs in Table 3 abolish high-affinity α -btx binding, and the small amount of specific binding observed at high α -btx concentrations suggests markedly reduced affinity. Alternatively, the mutations may have impaired folding or subunit assembly, reducing or preventing expression of AChRs on the cell surface. To determine whether the four mutant receptors express on the cell surface, we used patch-clamp electrophysiology to assess cell-surface expression, and also to look for changes in functional properties. For all four mutants that lack high-affinity α -btx binding, acetylcholine elicits single-channel currents that are very similar to those observed for the control $\alpha 7/5\text{HT}_3$ receptor (Figure 7). The open time histograms, fitted by the sum of three exponentials, are similar between the control $\alpha 7/5\text{HT}_3$ receptor and each of the four mutants. The closed time histograms contain five exponential components, the three briefest of which are similar between the control $\alpha 7/5\text{HT}_3$ receptor and the four mutants; time constants for the longest components differ among the four mutants, but one and possibly both long components depend on the number of receptors in the patch and thus are expected to vary among different patches. Thus the four α -btx-insensitive mutant receptors express on the cell surface and exhibit kinetic properties very similar to those of the control $\alpha 7/5\text{HT}_3$ receptor.

DISCUSSION

Atomic-resolution structural data, mutagenesis, radioligand binding and electrophysiological measurements reveal structural contributions to the high-affinity target-selective interaction between a long-chain α -neurotoxin and a nicotinic AChR. The results resolve an apparent paradox that, within a large contact surface area between α -btx and the $\alpha 7$ AChR, only one of five conserved aromatic residues that contact the toxin is essential for the high-affinity interaction [21]. In particular, the contribution of the essential Tyr¹⁸⁴ in $\alpha 7$ depends on the proximal residues Tyr¹⁹¹ and Phe¹⁸³, and these complementary residues also contribute to binding in a mutually interdependent manner. Furthermore, the concept of proximal interdependent residues explains α -btx insensitivity of neuronal AChRs containing $\alpha 2$ and $\alpha 3$ subunits. For these neuronal AChRs, the two residues that flank Tyr¹⁸⁴ differ from those in $\alpha 7$ and are interdependent in conferring low affinity for α -btx. For constructs containing loop C from the α -btx-insensitive $\alpha 4$ subunit, high affinity is maintained, whereas the rate and extent of α -btx dissociation is increased, indicating that additional structural determinants contribute to α -btx insensitivity of AChR subtypes containing $\alpha 4$. The overall findings highlight the importance of interdependent residue networks in contributing to high-affinity protein-protein interactions.

High-affinity binding of long-chain α -neurotoxins to nicotinic AChRs arises from very low rate constants of both association and dissociation [3,4]. Association of α -btx with $\alpha 7$ occurs at a rate some three orders of magnitude lower than that of a diffusion-controlled reaction, suggesting the rate-limiting steps include conformational selection of each binding partner. In $\alpha 7$, loop C adopts a range of conformations [21,32], and a fully open conformation may be required to allow enough room to accommodate finger II of α -btx. In solution, the Arg³⁶ and Phe³² side chains of α -btx adopt a range of conformations [14,15], and the guanidinium and aromatic moieties may need to come into close register to stably interact with Tyr¹⁸⁴. The interdependent residue networks identified in the present study contribute little to the rate of α -btx association, suggesting that the networks do not affect the large-scale conformational dynamics of the receptor target.

Dissociation of α -btx from $\alpha 7$ occurs in two steps, one progressing over tens of minutes, and a second over more than several hours. We find that the interdependent residue networks affect the rate of the fast dissociation step, with the paired mutations increasing the dissociation rate as much as 40-fold, whereas they do not affect the rate of the slow dissociation step. Furthermore, the paired mutations increase the magnitude of the fast

relative to the slow dissociation step, indicating that the stability of the initial complex determines the extent of formation of the stable complex. Thus, whereas the inter-residue interactions described in the present study determine the stability of the initial toxin–receptor complex, subsequent conformational rearrangements may be required to form the very stable complex. These slower rearrangements may occur subsequent to locking of loop C in its most outward conformation.

Previous studies established that sequence differences in loop C accounted for α -btx insensitivity in certain animals [11,12]. In particular, a glycosylation site at the position equivalent to Phe¹⁸³ accounts for invulnerability of the snake to α -btx, and glycosylation at a position equivalent to Glu¹⁸¹ accounts for the invulnerability of the mongoose. On the other hand, loop C from the hedgehog does not contain a glycosylation site, yet the hedgehog is partially resistant to α -btx [34]. In loop C of the hedgehog, isoleucine and alanine flank the essential Tyr¹⁸⁴, in contrast with phenylalanine and serine in animals most sensitive to α -btx. By altering the inter-residue network encompassing Tyr¹⁸⁴, these naturally occurring replacements apparently produce a subtle structural change that is nearly as effective as a glycosylation site in reducing toxin sensitivity.

An analogous interdependent residue network underlies α -btx insensitivity of $\alpha 2$ and $\alpha 3$ containing neuronal AChRs, which exhibit sequence variability at the positions flanking the essential Tyr¹⁸⁴ (Table 3). Because our studies were conducted with homomeric receptors with replacements in loop C, our conclusions require confirmation in heteromeric neuronal AChRs. However, previous studies of neuronal $\alpha 3$ AChRs suggest our results may extend to heteromeric AChRs. When the sequence YKHEIKYN, within loop C of the $\alpha 3$ subunit, was replaced by the sequence WKHWVYYT from *Torpedo californica* (residue equivalent to Tyr¹⁸⁴ is bold and underlined), and the construct co-expressed with neuronal β -subunits, heteromeric AChRs formed that were sensitive to α -btx [16,35]. Thus previous studies in heteromeric AChRs containing $\alpha 3$ are consistent with our findings.

After loop C of $\alpha 4$ was substituted into $\alpha 7$, high-affinity α -btx binding remained, but the rate and extent of fast dissociation increased. Thus, in AChRs containing $\alpha 4$, structures in addition to loop C further reduce sensitivity to α -btx. Contributions of the complementary subunits to α -btx binding were observed in muscle AChRs [36], and interactions between residues on adjacent β -strands of the complementary subunits contributed interdependently to α -conotoxin M1 binding [37]. Thus additional contributions to α -btx insensitivity of heteromeric $\alpha 4$ AChRs may arise from within the $\alpha 4$ subunit or the neuronal $\beta 2$ – $\beta 4$ subunits.

The structural principles elucidated in the present study clarify structural mechanisms of molecular recognition of AChRs by three-finger α -neurotoxins, including long-chain α -neurotoxins, κ -neurotoxins, short chain α -neurotoxins and non-conventional α -neurotoxins (Figure 8). Long-chain α -neurotoxins contain a disulfide bond within finger II, and share residues identical or chemically similar to Arg³⁶, Phe³² and Asp³⁰ of α -btx (Figure 8), suggesting that high-affinity binding of these α -neurotoxins depends on Tyr¹⁸⁴ and flanking residues in loop C. κ -Neurotoxins bind selectively to neuronal AChRs containing the $\alpha 3$ subunit [38], contain a disulfide bond within finger II, and also share residues identical or chemically similar to Arg³⁶, Phe³² and Asp³⁰ of α -btx (Figure 8). Although κ -neurotoxins form non-covalent dimers [39], finger II from each monomer extends from opposing ends of the dimer [40], and could interact with an AChR subunit interface. Although dimerization of κ -neurotoxins likely contributes to their affinity for neuronal AChRs [41], specialized residues flanking Tyr¹⁸⁴ (Figure 1) probably also contribute to κ -neurotoxin selectivity for AChRs containing $\alpha 3$. Short α -neurotoxins bind with high affinity to muscle, but not $\alpha 7$ AChRs [42], and lack both the disulfide bond in finger II and the four intervening residues.

Finger II, though shorter, contains three residues that are either identical or chemically similar to the triad Arg³⁶, Phe³² and Asp³⁰ in α -btx (Figure 8). The structure of erabutoxin a (PDB code 1QKD) shows that the three equivalent residues occupy positions similar to the residue triad in α -btx; this explains why short α -neurotoxins bind to muscle AChRs, whereas their inability to bind to $\alpha 7$ AChRs may arise from structural specialization between fingers I and II. In non-conventional α -neurotoxins, the amino acid sequences of finger II vary widely and usually lack the residue triad found in α -btx, which explains the low potency of the weak α -neurotoxin from *Naja kaouthia* [43]. However another non-conventional α -neurotoxin, candoxin, binds to both muscle and $\alpha 7$ AChRs [44], and its structure (PDB code 1JGK) shows that the arginine and glutamate residues (Figure 8) project from finger II and occupy positions similar to Arg³⁶ and Phe³² in α -btx. In summary, within the large surface area of contact between an α -neurotoxin and the AChR ligand-binding site, the interplay among a few residues, conserved and nonconserved, contributes to both high-affinity and subtype-selective molecular recognition.

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Abbreviations used

AChR	acetylcholine receptor
AChBP	acetylcholine-binding protein
α-btx	α -bungarotoxin
α-ctx	α -cobratoxin
HEK	human embryonic kidney
5HT_{3A}	5-hydroxytryptamine type 3A

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$\alpha 1$ - ..SVT**Y**SCCPDTPYLD
 $\alpha 2$ - ..SKK**Y**DCCAEI.YPD
 $\alpha 3$ - ..DIK**Y**NCCEEI.YPD
 $\alpha 4$ - ..TRK**Y**ECCAEI.YPD
 $\alpha 5$ - ..NRTDSCCW...YPY
 $\alpha 6$ - ..DIK**Y**NCCEEI.YTD
 $\alpha 7$ - ..ERF**Y**ECCKEP.YPD
 $\alpha 8$ - ..ELY**Y**ECCKEP.YPD
 $\alpha 9$ - ..VIS**Y**GCCSEP.YPD
 $\alpha 10$ - ..VL**T**YGCCSEP.YPD

Figure 1. Sequences of loop C in subtypes of human nicotinic receptor α -subunits
Tyr¹⁸⁴ is highlighted in bold.

Figure 2. Crystal structure of the complex between α -btx (purple) and the $\alpha 7$ /AChBP chimaera (cyan and orange)

PDB code 4HQP [21]. (a) Top view of the complex with the complementary subunit of one binding site highlighted in orange. (b) Close-up view as in (a) showing the principal (cyan) and complementary (orange) subunits. (c) Stereo view of key residues between the tip of finger II of α -btx (purple) and the principal (cyan) and complementary (orange) subunits of the $\alpha 7$ /AChBP chimaera.

Figure 3. Steady-state binding of ^{125}I -labelled α -btx to $\alpha 7/5\text{HT}_3$ receptors and the indicated mutants expressed in HEK-293 cells

See the Experimental section for more details. Curves through the data are fits of the Hill equation with parameters given in Table 1. $\alpha 7$, $\alpha 7/5\text{HT}_3$.

Figure 4. Binding time courses of 125 I-labelled α -btx at the indicated concentrations to $\alpha 7/5\text{HT}_3$ receptors and the indicated mutants expressed in HEK-293 cells

See the Experimental section for more details. Curves through the data are global fits of the bimolecular binding mechanism (see the main text) to the data with fitted parameters given in Table 2. RT, receptor-toxin complex.

Figure 5. Time courses of dissociation of ^{125}I -labelled α -btx from $\alpha 7/5\text{HT}_3$ receptors and the indicated mutants expressed in HEK-293 cells

See the Experimental section for more details. Curves through the data are fits of a single exponential plus a variable plateau to the data with fitted parameters in Table 2.

Figure 6. Determinants of target selectivity of α -btX determined from steady-state ^{125}I -labelled α -btX binding to $\alpha 7/5\text{HT}_3$ receptors with the indicated loop C replacements expressed in HEK-293 cells

See the Experimental section for more details. Curves through the data are fits of the Hill equation with parameters given in Table 3. Bold italic letters indicate substitutions.

Figure 7. Single-channel currents through $\alpha 7/5\text{HT}_3$ receptors with the indicated loop C replacements expressed in HEK-293 cells

See the Experimental section for more details. For each panel, a trace of single channel currents elicited by 1 mM acetylcholine is shown filtered at a bandwidth of 10 kHz with channel opening corresponding with upward deflections. Underneath each trace are open (left) and closed (right) time histograms plotted on a logarithmic time scale and fitted by the sum of exponentials; individual components are indicated by dashed curves and the sum by solid curves.

Long-chain α -neurotoxins:

α -bungarotoxin ..CYRKMWC**DAFCSSRGKVVELGC**..
 α -cobratoxin ..CYTKTWC**DAFCSI**RGKRVDLGC..
 lc-a ..CYLKSWD**DGTGFLKGNRLEFGC**..
 toxin 3 ..CYTKTWC**DAFCSI**RGKRVDLGC..
 LS III ..CYVKSWC**NAWCSSRGKVLEFGC**..

 κ -neurotoxins:

κ -bungarotoxin ..CFLKAQC **DKFCSI**RGPVIEQGC..
 κ 2-bungarotoxin ..CFLKVSC **EQFCPI**RGPVIEQGC..
 κ 3-bungarotoxin ..CFRKAQC **DNFCHS**RGPVIEQGC..
 κ -flavitoxin ..CFTKAFC **DRWCSS**RGPVIEQGC..

Short-chain α -neurotoxins:

Erabutoxin b ..CYHKQS-**D-F**---**RG**TIIERGC..
 Nmm I ..CYKKRWR**D-H**---**RG**YRTERGC..
 toxin- α ..CYKKVWR**D-H**---**RG**TIIERGC..

Non-conventional α -neurotoxins:

Candoxin ..CFKESW**E**---**AR**GTRIERGC..
 N. kaouthia WTX ..CFKKLHQRR---PLSWRYIRGC..

Figure 8. Sequence alignment of Finger II in 3-finger α -neurotoxins
 Key residues are bold. α -Neurotoxin sequences are from [38,44,45].

Table 1
Binding free energy and inter-residue coupling free energy determined from steady-state ^{125}I -labelled α -btx binding

For each fitted or calculated value, mean $\pm$ S.E.M. is given.

Mutant	K_d (nM)	n_H	ΔG (kcal/mol)	$\Delta\Delta G$ (kcal/mol)
α 7/5HT ₃	8.7 $\pm$ 0.56	1.96 $\pm$ 0.10	0.0 $\pm$ 0.017	–
Y184F	7.8 $\pm$ 0.36	1.63 $\pm$ 0.05	0.066 $\pm$ 0.022	–
Y191T	9.7 $\pm$ 0.44	1.81 $\pm$ 0.07	–0.065 $\pm$ 0.021	–
Y91T	7.9 $\pm$ 0.46	1.87 $\pm$ 0.10	0.058 $\pm$ 0.025	–
W145T	9.3 $\pm$ 0.50	1.73 $\pm$ 0.06	–0.04 $\pm$ 0.024	–
W53T	11.5 $\pm$ 0.60	1.66 $\pm$ 0.04	–0.17 $\pm$ 0.025	–
F183T	7.7 $\pm$ 0.44	1.90 $\pm$ 0.09	0.073 $\pm$ 0.023	–
Y191T/F183T	154 $\pm$ 5.3	1.96 $\pm$ 0.02	–1.72 $\pm$ 0.020	–1.73 $\pm$ 0.028
Y191T/Y184F	51.2 $\pm$ 0.52	1.27 $\pm$ 0.07	–1.06 $\pm$ 0.033	–1.06 $\pm$ 0.038
Y184F/F183T	28.8 $\pm$ 2.6	1.51 $\pm$ 0.09	–0.72 $\pm$ 0.029	–0.86 $\pm$ 0.036
F183T/W53T	51 $\pm$ 5.4	1.57 $\pm$ 0.11	–1.06 $\pm$ 0.034	–0.85 $\pm$ 0.041
Y184F/W53T	32 $\pm$ 2.7	1.63 $\pm$ 0.09	–0.78 $\pm$ 0.028	–0.56 $\pm$ 0.036
Y191T/W53T	30 $\pm$ 3.4	1.37 $\pm$ 0.09	–0.74 $\pm$ 0.036	–0.38 $\pm$ 0.043
Y91T/W53T	12.8 $\pm$ 1.7	1.55 $\pm$ 0.06	–0.23 $\pm$ 0.025	0.24 $\pm$ 0.036
W145T/W53T	15.7 $\pm$ 3.3	1.60 $\pm$ 0.10	–0.35 $\pm$ 0.030	0.12 $\pm$ 0.039
Y191T/W145T	10.0 $\pm$ 0.35	1.72 $\pm$ 0.04	–0.084 $\pm$ 0.020	0.022 $\pm$ 0.032
Y191T/Y91T	15.4 $\pm$ 1.4	1.72 $\pm$ 0.11	–0.34 $\pm$ 0.029	–0.34 $\pm$ 0.037
Y91T/F183T	11.4 $\pm$ 0.66	1.68 $\pm$ 0.07	–0.16 $\pm$ 0.026	–0.29 $\pm$ 0.036
Y91T/W145T	7.4 $\pm$ 0.49	1.44 $\pm$ 0.06	0.097 $\pm$ 0.025	0.079 $\pm$ 0.035
Y91T/Y184F	11.4 $\pm$ 0.31	1.68 $\pm$ 0.023	0.160 $\pm$ 0.024	0.37 $\pm$ 0.037
W145T/F183T	9.2 $\pm$ 0.35	1.80 $\pm$ 0.05	–0.034 $\pm$ 0.020	–0.067 $\pm$ 0.031
W145T/Y184F	8.1 $\pm$ 0.50	1.44 $\pm$ 0.06	0.043 $\pm$ 0.024	0.017 $\pm$ 0.033

Binding free energy and inter-residue coupling free energy determined from the kinetics of ^{125}I -labelled α -btx binding

In the first two columns, k_{+1} and k_{-1} are the values obtained by Dynafit analyses of the data in Figure 4. In the third and fourth columns, k_{-1} and Plateau (percentage of highly stable binding) were determined by analyses of the data in Figure 5 (see the main text). For each fitted rate constant a standard error is expressed as a percentage of the fitted value. nd, not determined.

Mutant	k_{+1} ($\text{M}^{-1}\text{s}^{-1}$) fitted	k_{-1} (s^{-1}) fitted	k_{-1} (s^{-1}) measured	Plateau	K_d (nM)	ΔG (kcal/mole)	$\Delta\Delta G$ (kcal/mole)
α 7/5HT ₃	9.2×10^4 (2.7 %)	8.8×10^{-4} (7.2 %)	3.5×10^{-4} (8.6 %)	78 ± 1.1	3.8 ± 0.34	0.0 ± 0.05	–
Y191T	1.04×10^5 (2.8 %)	nd	8.1×10^{-4} (4.4 %)	55 ± 1.7	7.8 ± 0.41	0.43 ± 0.032	–
F183T	9.6×10^4 (4.7 %)	nd	7.5×10^{-4} (12.5 %)	71 ± 0.7	7.8 ± 1.04	0.43 ± 0.08	–
Y184F	1.5×10^5 (1.8 %)	nd	5.7×10^{-4} (2.2 %)	51 ± 1.9	3.8 ± 0.11	0.0 ± 0.02	–
Y191T/F183T	6.2×10^4 (7.0 %)	1.3×10^{-2} (5.9 %)	1.4×10^{-2} (5.8 %)	8.2 ± 0.6	210 ± 19	2.41 ± 0.05	-1.54 ± 0.12
Y191T/Y184F	9.5×10^4 (5.0 %)	5.3×10^{-3} (5.8 %)	6.8×10^{-3} (6.7 %)	17 ± 1.5	56 ± 4.3	1.61 ± 0.05	-1.18 ± 0.08
Y184F/F183T	1.2×10^5 (3.8 %)	4.7×10^{-3} (4.8 %)	6.2×10^{-3} (6.5 %)	15 ± 1.5	39 ± 2.4	1.40 ± 0.04	-0.97 ± 0.11

Table 3
¹²⁵I-labelled α -btx binding and dissociation for mutant $\alpha 7/5HT_{3A}$ receptors with different substitutions of loop C

Columns 1–3 give analyses of data from steady-state ¹²⁵I-labelled α -btx binding as in Figure 5, whereas columns 4 and 5 give analyses of data from ¹²⁵I-labelled α -btx dissociation as in Figure 6, where τ_{diss} is the time constant for fast α -btx dissociation, and Plateau is the percentage of highly stable binding (see text). For each fitted value a standard error is given. Residues in bold and italic indicate mutations. nd, not defined.

Loop C sequence	K_d (nM)	n_H	K_{mut}/K_{wt}	τ_{diss} (min)	Plateau
ERFYECCKEPYP ($\alpha 7$)	10.1 $\pm$ 0.6	1.6 $\pm$ 0.08	1.0	43 $\pm$ 3.3	78 $\pm$ 0.4
ERFY N CKKEPYP	15.8 $\pm$ 1.0	1.4 $\pm$ 0.05	1.6	–	–
ER K YECCKEPYP	15.9 $\pm$ 1.0	1.6 $\pm$ 0.06	1.6	16 $\pm$ 1.1	29 $\pm$ 1.0
ER K N CCKEPYP	1010 $\pm$ 630	1.0 $\pm$ 0.06	100	–	–
SKKYDCCAEIYP ($\alpha 2$)	7200 $\pm$ 5800	0.7 $\pm$ 0.05	712	–	–
SKKY E CCAEIYP	12.5 $\pm$ 0.6	1.5 $\pm$ 0.05	1.2	14 $\pm$ 0.4	19 $\pm$ 0.5
SK F YDCCAEIYP	7.8 $\pm$ 0.5	2.1 $\pm$ 0.12	0.77	–	–
SK F E CCAEIYP	9.6 $\pm$ 0.6	2.1 $\pm$ 0.11	0.95	57 $\pm$ 17	83 $\pm$ 1.3
DIKYNCC E IYP ($\alpha 3$)	2000 $\pm$ 350	0.9 $\pm$ 0.03	198	–	–
DIKY E CC E IYP	14.0 $\pm$ 0.5	1.8 $\pm$ 0.05	1.4	28 $\pm$ 1.7	39 $\pm$ 0.7
D I FYNCC E IYP	12.9 $\pm$ 0.6	1.9 $\pm$ 0.07	1.3	–	–
D I F E CC E IYP	9.8 $\pm$ 0.4	2.1 $\pm$ 0.08	0.97	189 $\pm$ 172	83 $\pm$ 7.3
WVFYSCCPTTPYL ($\alpha 1$)	4.8 $\pm$ 0.4	1.3 $\pm$ 0.06	0.48	–	–
WV K YNCCPTTPYL	>5200	nd	>515	–	–
WVFYSCC E E IYP	3.9 $\pm$ 0.3	1.9 $\pm$ 0.11	0.39	–	–
TRKYECCAEIYPD ($\alpha 4$)	12.5 $\pm$ 0.6	1.6 $\pm$ 0.06	1.2	17 $\pm$ 0.6	19 $\pm$ 0.5
TR F YECCAEIYPD	9.3 $\pm$ 0.5	1.8 $\pm$ 0.8	0.92	–	–
TRKY N CCAEIYPD	2600 $\pm$ 800	0.8 $\pm$ 0.04	257	–	–