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Key structural determinants in the agonist binding loops of human $\beta 2$ and $\beta 4$ nicotinic acetylcholine receptor subunits contribute to $\alpha 3\beta 4$ subtype selectivity of α -conotoxins

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Abstract

α -Conotoxins represent a large group of pharmacologically active peptides that antagonize nicotinic acetylcholine receptors (nAChRs). The $\alpha 3\beta 4$ nAChR, a predominant subtype in the peripheral nervous system, has been implicated in various pathophysiological conditions. As many α -conotoxins have multiple pharmacological targets, compounds specifically targeting individual nAChR subtypes are needed. In this study, we performed mutational analyses to evaluate the key structural components of human $\beta 2$ and $\beta 4$ nAChR subunits that determine α -conotoxin selectivity for $\alpha 3\beta 4$ nAChR. α -Conotoxin RegIIA was used to evaluate the impact of non-conserved human $\beta 2$ and $\beta 4$ residues on peptide affinity. Two mutations, $\alpha 3\beta 2$ [T59K] and $\alpha 3\beta 2$ [S113R], strongly enhanced RegIIA affinity compared with wild-type $\alpha 3\beta 2$, as seen by substantially increased inhibitory potency and slower off-rate kinetics. Opposite point mutations in $\alpha 3\beta 4$ had the contrary effect, emphasizing the importance of loop D residue 59 and loop E residue 113 as determinants for RegIIA affinity. Molecular dynamics simulation revealed the side chains of $\beta 4$ Lys⁵⁹ and $\beta 4$ Arg¹¹³ formed hydrogen bonds with RegIIA loop 2 atoms, whereas the $\beta 2$ Thr⁵⁹ and $\beta 2$ Ser¹¹³ side chains were not long enough to form such interactions. Residue $\beta 4$ Arg¹¹³ has been identified for the first time as a crucial component facilitating antagonist binding. Another α -conotoxin, AulB, exhibited low activity at human $\alpha 3\beta 2$ and $\alpha 3\beta 4$ nAChRs. Molecular dynamics simulation indicated the key interactions with the β subunit are different to RegIIA. Taken together, these data elucidate the interactions with specific individual β subunit residues that critically determine affinity and pharmacological activity of α -conotoxins RegIIA and AulB at human nAChRs.

Keywords

binding, agonist, determinants, structural, key, acetylcholine, receptor, subtype, selectivity, nicotinic, $\alpha 3\beta 4$, subunits, contribute, $\beta 4$, α -conotoxins, $\beta 2$, human, loops

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Key Structural Determinants in the Agonist Binding Loops of Human $\beta 2$ and $\beta 4$ Nicotinic Acetylcholine Receptor Subunits Contribute to $\alpha 3\beta 4$ Subtype Selectivity of α -Conotoxins^{*[5]}

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α -Conotoxins represent a large group of pharmacologically active peptides that antagonize nicotinic acetylcholine receptors (nAChRs). The $\alpha 3\beta 4$ nAChR, a predominant subtype in the peripheral nervous system, has been implicated in various pathophysiological conditions. As many α -conotoxins have multiple pharmacological targets, compounds specifically targeting individual nAChR subtypes are needed. In this study, we performed mutational analyses to evaluate the key structural components of human $\beta 2$ and $\beta 4$ nAChR subunits that determine α -conotoxin selectivity for $\alpha 3\beta 4$ nAChR. α -Conotoxin RegIIA was used to evaluate the impact of non-conserved human $\beta 2$ and $\beta 4$ residues on peptide affinity. Two mutations, $\alpha 3\beta 2$ [T59K] and $\alpha 3\beta 2$ [S113R], strongly enhanced RegIIA affinity compared with wild-type $\alpha 3\beta 2$, as seen by substantially increased inhibitory potency and slower off-rate kinetics. Opposite point mutations in $\alpha 3\beta 4$ had the contrary effect, emphasizing the importance of loop D residue 59 and loop E residue 113 as determinants for RegIIA affinity. Molecular dynamics simulation revealed the side chains of $\beta 4$ Lys⁵⁹ and $\beta 4$ Arg¹¹³ formed hydrogen bonds with RegIIA loop 2 atoms, whereas the $\beta 2$ Thr⁵⁹ and $\beta 2$ Ser¹¹³ side chains were not long enough to form such interactions. Residue $\beta 4$ Arg¹¹³ has been identified for the first time as a crucial component facilitating antagonist binding. Another α -conotoxin, AuIB, exhibited low activity at human $\alpha 3\beta 2$ and $\alpha 3\beta 4$ nAChRs. Molecular dynamics simulation indicated the key interactions with the β subunit are different to RegIIA. Taken together, these data elucidate the interactions with specific individual β subunit residues that critically determine affinity and pharmacological activity of α -conotoxins RegIIA and AuIB at human nAChRs.

Neuronal nicotinic acetylcholine receptors (nAChR)³ are ligand-gated ion channels endogenously activated by acetylcholine (ACh). Presynaptic nAChRs in the central nervous system play an important role in synaptic transmission, as they mediate the release of various neurotransmitters (1). Postsynaptic nAChRs are involved in fast excitatory transmission, and non-synaptic nAChRs modulate many neurotransmitter systems by influencing neuronal excitability (2). The widespread expression and functional diversity makes nAChRs key components in numerous physiological functions of the central and peripheral nervous systems such as learning, memory, attention, motor control, synaptic plasticity, and analgesia (2, 3). Based on these versatile physiological roles, any dysfunction of nAChR subtypes contributes to various disease states, including epilepsy, schizophrenia, Parkinson's disease, depression, autism, Alzheimer's disease, and addiction (2–5).

Functional neuronal nAChRs are composed of five transmembrane-spanning subunits combining α ($\alpha 2$ – $\alpha 10$) and β ($\beta 2$ – $\beta 4$) subunits. Combinations of different α and β subunits yields a variety of heteromeric receptors with individual physiological roles and pharmacological profiles (6, 7). Functional neuronal nAChRs containing an $\alpha 2$, $\alpha 3$, $\alpha 4$, or $\alpha 6$ subunit also require a $\beta 2$ or $\beta 4$ subunit. The β subunit in these nAChR subtypes influences pharmacological properties including agonist efficacy, desensitization kinetics, and Ca²⁺ permeability (3).

The ligand binding site of nAChRs lies at the extracellular interface between α and β subunits, with the α subunit contributing the principal (+) face and the β subunit contributing the complementary (–) face. The ligand binding sites of nAChR subtypes are structurally very similar and hence the residues involved in agonist binding are considerably conserved. Therefore, the development of highly subtype-specific competitive ligands is a difficult ongoing task (6).

α -Conotoxins are a large group of disulfide-bonded peptides isolated from the venom of carnivorous marine *Conus* snails. They have been found to be specific competitive ligands of

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[5] This article contains supplemental Fig. S1.

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³ The abbreviations used are: nAChR, nicotinic acetylcholine receptor; ACh, acetylcholine; AcM, acetamidomethyl; ECD, extracellular domain; Fmoc, N-(9-fluorenyl)-methoxycarbonyl; MD, molecular dynamics; CI, confidence interval.

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nAChRs, inhibiting ACh-evoked currents in a concentration-dependent manner. All α -conotoxins contain two conserved disulfide bridges and can be subclassified by the number of residues between the cysteines forming the disulfide bonds (e.g. $\alpha 4/3$, $\alpha 4/4$, and $\alpha 4/7$) (8). Individual α -conotoxins have their own selectivity profile and can discriminate between different nAChR subunit combinations and stoichiometries (9, 10).

To date, a number of highly selective α -conotoxins potentially inhibiting the $\alpha 3\beta 2^*$ subtype have been identified (* indicates the presence of potential other subunits) (8). However, few probes specifically inhibiting single $\alpha 3$ -containing nAChR subtypes exist (6). The $\alpha 3\beta 4$ nAChR is the predominant subtype in the peripheral nervous system and medial habenula of the brain and it has been shown to be involved in several pathophysiological disease conditions such as lung cancer, nicotine addiction, and drug abuse (3, 11, 12). Therefore, $\alpha 3\beta 4$ is a promising therapeutic target and pharmacologically active substances modulating this nAChR subtype are sought-after.

The first $\alpha 3\beta 4$ selective α -conotoxin described was $\alpha 4/6$ -conotoxin AuIB, which inhibits rat $\alpha 3\beta 4$ with an IC_{50} of 0.75 μM , whereas activity at other subtypes was reported as >100-fold lower (13). Another $\alpha 4/6$ -conotoxin, TxID from *Conus textile*, was recently described as the most potent $\alpha 3\beta 4^*$ nAChR-inhibiting α -conotoxin, however, besides $\alpha 3\beta 4$ it also inhibits $\alpha 6/\alpha 3\beta 4$ with 7.5-fold less potency (14).

$\alpha 4/7$ -Conotoxin RegIIA from *Conus regius* has been reported to potentially inhibit $\alpha 3\beta 4$ besides $\alpha 3\beta 2$ and $\alpha 7$ nAChRs (15). Interestingly, a non-natural analogue of RegIIA in which two residues in loop 2 were exchanged by alanines exhibited an enhanced selectivity for the $\alpha 3\beta 4$ subtype compared with the wild-type toxin (16). Furthermore, we have previously shown that the selectivity profile of RegIIA differs between nAChRs of different species, as RegIIA was equipotent at the rat (r) and human (h) $\alpha 3\beta 4$ subtypes but significantly less active at $\alpha 3\beta 2$ compared with $\alpha 3\beta 2$. This potency difference could be mapped to a single non-conserved Glu¹⁹⁸ on the $\alpha 3$ subunit (proline in human) (17).

Here, we investigated the molecular determinants for nAChR subtype selectivity of α -conotoxins, especially for the $\alpha 3\beta 4$ subtype. We tested residues, which are non-conserved between the homologous $\beta 2$ and $\beta 4$ subunits, for their impact on α -conotoxin affinity and activity using β subunit chimeras and point mutations. α -Conotoxin RegIIA was used as a probe for a major part of this study. We aimed to exhibit key β subunit residues of the ligand-binding interface that profoundly and subtype specifically affect both RegIIA sensitivity and wash-off kinetics.

Experimental Procedures

Peptide Synthesis— α -Conotoxin RegIIA was synthesized as described previously (15). The α -conotoxin AuIB was assembled on Rink amide methylbenzhydrylamine resin using solid-phase peptide synthesis with a neutralization/2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate activation procedure for Fmoc (*N*-(9-fluorenyl)-methoxycarbonyl) chemistry. Cleavage was achieved by treatment with 88:2:5:5 (v:v) ratio of trifluoroacetic acid/triisopropylsilane/phenol/water at room temperature (20–25 °C) for 2 h. Trifluo-

oroacetic acid was evaporated at low pressure in a rotary evaporator. Peptides were precipitated with ice-cold ether, filtered, dissolved in 50% buffer A/B (buffer A consists of 99.95% H₂O, 0.05% trifluoroacetic acid and buffer B consists of 90% CH₃CN, 10% H₂O, 0.045% trifluoroacetic acid), and lyophilized. Crude peptides were purified by RP-HPLC on a Phenomenex C₁₈ column using a gradient of 0–50% of buffer B for 50 min, with the eluent monitored at 214/280 nm. Electrospray mass spectrometry confirmed the molecular mass of the peptides before they were pooled and lyophilized for oxidation.

The four cysteines in the peptides were selectively oxidized in two steps to yield the globular conformation. This was achieved by incorporating Fmoc-Cys-acetamidomethyl (Acm)-OH at positions 2 and 8 of the amino acid sequence. In the first step, the two non-protected cysteines were oxidized in 0.1 M NH₄HCO₃ (pH 8–8.5) at a concentration of 0.5 mg/ml, and the mixture was stirred at room temperature for 48 h. The oxidized peptides were then purified and lyophilized as previously outlined. In the second step, the Acm-protected cysteines were oxidized by dissolving the peptides in iodine solution filled at 1 mg/ml, and the mixture was stirred for 35 min. After two rounds of oxidation, peptides were purified by RP-HPLC using a gradient of 0–80% buffer B over 180 min. Analytical RP-HPLC and electrospray mass spectrometry were used to confirm the purity and molecular mass of the synthesized peptides.

Protein Sequence Alignment—Protein sequences of h $\beta 2$ and h $\beta 4$ nAChR subunit ECDs (RefSeq accession numbers NP_000739 and NP_000741, respectively) were aligned using CLC Viewer 7 software (CLC bio, Aarhus, Denmark). Residues were numbered according to the mature protein sequences.

Site-directed Mutagenesis—Plasmid DNAs encoding human and rat $\alpha 3$, $\beta 2$, and $\beta 4$ nAChR subunits were subcloned into the pT7TS *Xenopus* expression vector (Addgene plasmid 17091) as described previously (17). Two chimeric receptors consisting of h $\beta 2$ backbone and portions of the N-terminal extracellular domain (ECD) (N-terminal T1 to loop D Glu⁶³ and a lesser conserved Lys⁷⁰-His⁸⁶ segment between loops D and E) replaced with the corresponding h $\beta 4$ sequences, were generated by overlap PCR and molecular cloning. Additional mutants of human β subunit loops D, E and F (Table 1), as well as rat $\beta 2$ [T59K] were engineered using the Geneart Site-directed Mutagenesis System (Invitrogen). All point mutations were confirmed by DNA sequencing (Australian Genome Research Facility, Melbourne, Australia).

Electrophysiological Recordings in *Xenopus* Oocytes and Data Analysis—RNA preparation, *Xenopus laevis* oocyte preparation, and expression of nAChR subunits in oocytes were performed as described previously (18).

Membrane currents from *Xenopus* oocytes were recorded using a single channel two-electrode voltage clamp setup (virtual ground circuit) with a GeneClamp 500B amplifier (Molecular Devices, Sunnyvale, CA) as described previously (17). Briefly, oocytes were continuously perfused with ND96 solution containing (in mM) 96 NaCl, 2 KCl, 1.8 CaCl₂, 1 MgCl₂, and 5 HEPES (pH 7.4) at 2 ml/min. ACh or ACh plus toxin was applied for ~2 s using a manual HPLC injection module. The effect of the peptides on ACh-evoked currents was defined as ACh plus peptide peak current amplitude relative to the aver-

age peak current amplitude of 3–5 control ACh applications (300 μM for $\alpha 3\beta 4$ and 50 μM for all other nAChR subtypes) recorded before preincubation with the peptides (technical replicates of ACh application). Perfusion was switched off during preincubation with the peptide. Concentration-response curves were fitted by unweighted nonlinear regression to the logistic equation,

$$E_x = E_{\text{max}} X^{n_H} / (X^{n_H} + \text{IC}_{50}^{n_H}) \quad (\text{Eq. 1})$$

where E_x is the response, X is the antagonist concentration, E_{max} is the maximal response, n_H is the Hill coefficient, and IC_{50} is the antagonist concentration that gives 50% inhibition of the agonist response.

Measurement of the recovery from block (k_{off}) by the peptides was carried out by incubating the oocyte with the respective peptide for 5 min followed by repeated ACh applications at the indicated time points while continuously perfusing the oocyte with ND96 solution at 2 ml/min. Approximate half-maximal excitatory concentrations (EC_{50}) of ACh were determined and used in the wash-off kinetic experiments.

All electrophysiological data were pooled ($n = 3$ –9 for each data point) and represent arithmetic mean \pm 95% confidence intervals (95% CI) of the fit. Each oocyte was tested only once with a given peptide concentration, therefore n indicates the number of oocytes used for generating each data point (biological replicates). Curves were fitted and statistics calculated using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA).

Homology Modeling—Models of RegIIA/AuIB-bound $\alpha 3\beta 2$ and $\alpha 3\beta 4$ nAChRs were built using Modeler (version 9v12), as described previously (19). The sequences of human $\alpha 1$, $\alpha 3$, $\alpha 4$, $\alpha 6$, $\alpha 7$, $\alpha 9$, $\alpha 10$, $\beta 2$, $\beta 3$, and $\beta 4$ nAChR subunits were retrieved from the Uniprot database (20). The crystal structures of *Aplysia californica* AChBP in complex with α -conotoxin PnIA[A10L,D14K] (PDB code 2BR8) (21), and the ECDs of mouse $\alpha 1$ (PDB code 2QC1) (22) and human $\alpha 9$ (PDB code 4D01) (23) subunits were used as templates to model the α -conotoxin-bound nAChR complexes (200 models each). Models with the lowest DOPE score (24) were selected for further structural refinement using molecular dynamics simulations.

Protonation State Predictions—The protonation states of amino acids His, Asp, and Glu were predicted using the PropKa 3.1 method (25). The predictions were made for all 200 homology models generated by Modeler and the protonation states predicted for the majority of models were considered in the starting models for MD simulations.

Molecular Dynamics (MD) Simulations—MD simulations were performed using the AMBER 12 package (26) and ff12SB force field (27). The above α -conotoxin-nAChR complexes were solvated in a truncated octahedral periodic box with TIP3P water molecules, and neutralized using sodium ions. Prior to MD simulations, 2000 steps of steepest descent minimization and 3000 steps of conjugate gradient minimization were performed with the solute restrained using a harmonic force with a 100 kcal/mol·Å² spring constant. After the first round of minimization, the entire system was minimized with-

out position restraints. The minimized systems were gradually heated from 50 to 300 K over 100 ps using NVT ensemble with the solute atoms restrained to their positions by harmonic forces with a spring constant of 5 kcal/mol·Å². Simulations were then switched to NPT ensemble, and spring constants of the restraints were gradually decreased from 5 to 0 kcal/mol·Å² over 100 ps. In the production phase, 50-ns MD simulations were carried out with the temperature and pressure maintained at 300 K and 1 bar, respectively.

In all simulations, all bonds involving hydrogen atoms were constrained with the SHAKE algorithm and the time step was 2 fs (28). The particle-mesh Ewald method was used to treat long-range electrostatic interactions (29).

Results

Two Single Non-conserved Residues in Loops D and E of the Human $\beta 2$ and $\beta 4$ nAChR Subunit ECDs Determine the Differential Sensitivity of $\alpha 3\beta 2$ and $\alpha 3\beta 4$ to α -Conotoxin RegIIA—To identify potential structural determinants responsible for the lower potency of α -conotoxin RegIIA at the $\alpha 3\beta 2$ subtype relative to $\alpha 3\beta 4$, we compared the amino acid sequences of the N-terminal ECDs containing the agonist binding domains of each subtype. Pairwise alignments of mature protein sequences of h $\beta 2$ and h $\beta 4$ lacking the signal peptide revealed an overall homology of 70% (149 of the 213 residues in the ECD are homologous) (Fig. 1A). Interestingly, the amino acids contributing to the complementary face of the ACh-binding loops that correspond to the previously identified homologous loops of the $\alpha 7$ subunit (30) are among the lesser conserved regions in the sequence. Other less conserved regions include the N terminus preceding loop D and the region from amino acids 70 to 86 (Fig. 1A). Homology modeling and overlay of the $\alpha 3\beta 2$ and $\alpha 3\beta 4$ intersubunit interfaces revealed the side chain differences between these non-conserved residues on the $\beta 2$ and $\beta 4$ subunits (Fig. 1B). These differences could account for the α -conotoxin nAChR subtype selectivity.

From electrophysiological recordings of oocyte-expressed nAChRs, RegIIA exhibited an IC_{50} of 45.6 nM (95% CI 31.5–65.9 nM; $n_H = 0.9$) at the $\alpha 3\beta 4$ subtype, whereas an IC_{50} of 132.4 nM (95% CI 109.8–159.7 nM; $n_H = 1.5$) was observed at the $\alpha 3\beta 2$ subtype (Fig. 2, A and B, and Table 1). To investigate if the less conserved sequences in the ECDs of the β -subunits determine the specificity of the peptide, we generated chimeric h $\beta 2$ - $\beta 4$ subunits, co-expressed them with wild-type h $\alpha 3$, and tested the effect of the peptide on ACh-evoked current amplitudes. The first chimeric subunit consisted of h $\beta 2$ in which the whole N-terminal region including loop D was replaced by the respective sequence of h $\beta 4$ (mutant h $\alpha 3\beta 2$ [1–63 $\beta 4$]) (Fig. 1A). Although the N-terminal region that precedes loop D is overall less conserved between the receptors, the loop itself only differs in one amino acid, the residue at position 59 (Lys in h $\beta 4$, Thr in h $\beta 2$). Mutant h $\alpha 3\beta 2$ [1–63 $\beta 4$] exhibited a similar sensitivity for RegIIA compared with wild-type h $\alpha 3\beta 2$ with an IC_{50} of 159.2 nM (95% CI 113.0–224.4; $n_H = 1.4$) (Fig. 2E, Table 1).

A refined mutation in which only the single non-conserved Thr residue in loop D was exchanged to the corresponding Lys residue in h $\beta 4$ (mutant h $\alpha 3\beta 2$ [T59K]) exhibited a concentration-response curve with an IC_{50} of 49.4 nM (95% CI 33.4–73.1;

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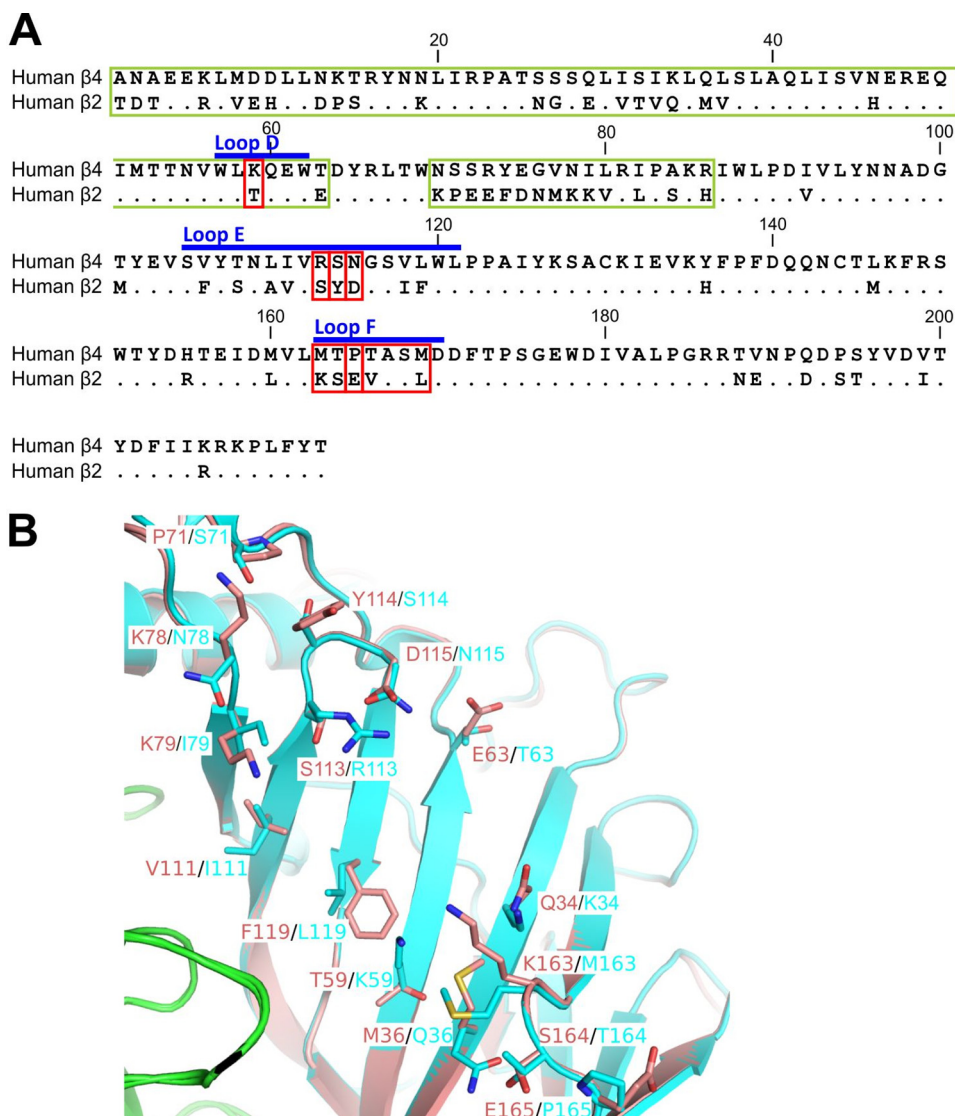


FIGURE 1. Sequence and structural comparison between human $\beta 2$ and $\beta 4$ nAChR ECDs. *A*, amino acid sequence alignment of the N-terminal ECDs of human $\beta 2$ and $\beta 4$ nAChR subunits shows 70% identity between the sequences (149 of the 213 residues homologous) and reveals several non-conserved residues in the ACh binding loops. Conserved residues are indicated with *dots*. Residues that were mutated to the opposite β subunit residue in this study are framed with *red lines*. Longer sequences that were replaced in h $\beta 2$ with the sequences of h $\beta 4$ are framed with *green lines*. *Blue bars* indicate ACh binding domain loops D, E, and F of the complementary interface (34). *B*, overlay of the $\alpha 3\beta 2$ and $\alpha 3\beta 4$ inter-subunit interfaces to emphasize the overall high structural similarity between them. The $\alpha 3(+)$ interface is shown in *green*, $\beta 2(-)$ in *pink*, and $\beta 4(-)$ in *cyan*. Non-conserved residues from the complementary $\beta 2(-)$ and $\beta 4(-)$ subunits, respectively, are shown as licorice models and labeled.

$n_H = 1.2$), which was significantly shifted to the left compared with wild-type $\alpha 3\beta 2$ and resembled the $\alpha 3\beta 4$ concentration-response curve (Fig. 2, *A* and *C*, and Table 1). Thus, the higher RegIIA sensitivity observed with $\alpha 3\beta 4$ can be (majorly) attributed to loop D. Conversely, the opposite mutation in h $\beta 4$ (mutant $\alpha 3\beta 4$ [K59T]) led to a severe loss in sensitivity to RegIIA, represented by an IC_{50} of 2795.1 nM (95% CI 2346.6–3329.3; $n_H = 1.4$) (Fig. 2, *B* and *D*, and Table 1) hence, confirming Lys⁵⁹ as a key determinant for human nAChR β subunit sensitivity to RegIIA.

Similarly, the lesser conserved region between loops D and E (residues 70 to 86) of h $\beta 2$ was replaced with the $\beta 4$ sequence (mutant $\alpha 3\beta 2$ [70–86 $\beta 4$]) and tested in oocytes. However, this mutation did not decrease the IC_{50} compared with wild-type $\alpha 3\beta 2$ but rather resulted in considerably lowered potency of the peptide possibly because of structural effects (Fig. 2*E*,

Table 1). Therefore, it was excluded as a factor for the $\beta 2-$ and $\beta 4-$ -specific differences in sensitivity to RegIIA.

The non-conserved residues Arg¹¹³, Ser¹¹⁴, and Asn¹¹⁵ of h $\beta 4$ were replaced with the respective residues of h $\beta 2$ (mutant $\alpha 3\beta 4$ [113–115 $\beta 2$]) to analyze the contribution of loop E for RegIIA affinity. This mutant considerably shifted the concentration-response curve for RegIIA to the right ($IC_{50} = 1056.5$ nM, 95% CI 924.9–1206.9 nM; $n_H = 1.1$). Contribution of any of the three residues to the shift was further dissected with point mutations in h $\beta 4$. Of these, only mutant $\alpha 3\beta 4$ [R113S] profoundly shifted RegIIA IC_{50} to the high nanomolar range (805.3 nM, 95% CI 554.7–1169.0 nM; $n_H = 0.9$) (Fig. 2, *B*, *D*, and *E*, and Table 1) compared with little contribution of mutations S114Y and N115D (IC_{50} of 131.2 nM, 95% CI 112.7–152.7 nM, $n_H = 1.3$, and IC_{50} of 275.0 nM, 95% CI 221.3–341.5 nM, $n_H = 1.6$, respectively). The opposite mutation, $\alpha 3\beta 2$ [S113R], exhibited a con-

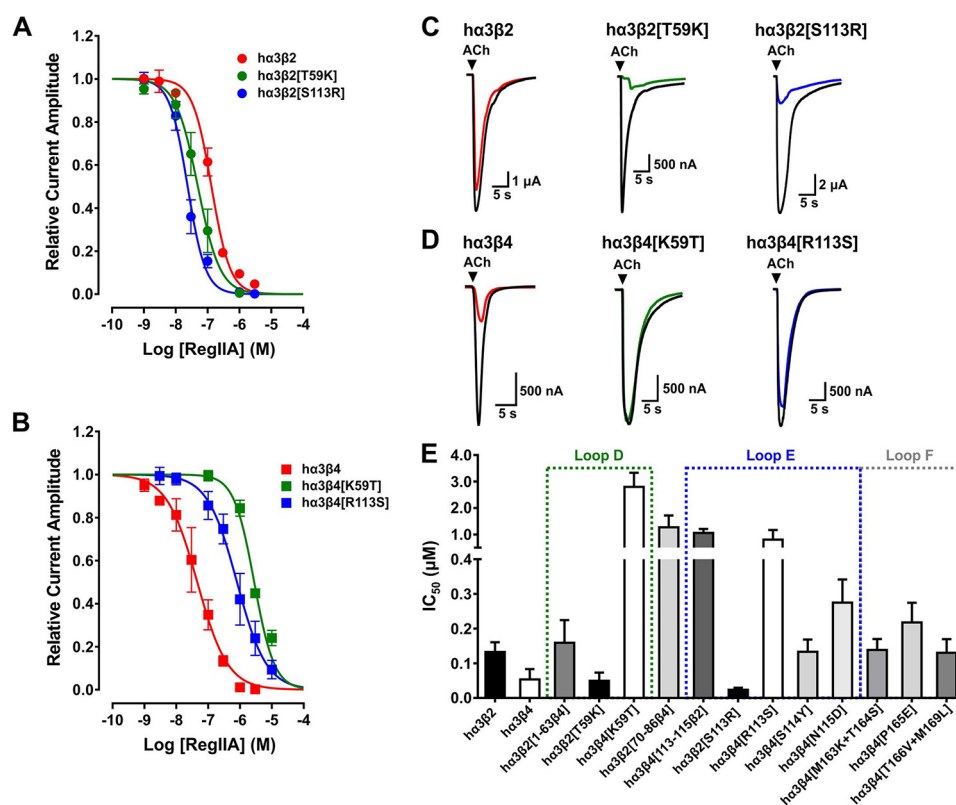


FIGURE 2. The non-homologous residues at position 59 (Lys in h $\beta 4$, Thr in h $\beta 2$) and 113 (Arg in h $\beta 4$, Ser in h $\beta 2$) of the human β subunits are the key residues determining the selectivity profile in inhibitory potency of α -conotoxin RegIIA. *A* and *B*, concentration-response curves for RegIIA inhibition of wild-type and mutant human $\alpha 3\beta 2$ (*A*) and $\alpha 3\beta 4$ nAChRs (*B*). The two $\alpha 3\beta 2$ mutants exhibited a shift of the curve to the left compared with wild-type $\alpha 3\beta 2$ nAChR, indicating an increase in affinity to the peptide, whereas the opposite mutants of $\alpha 3\beta 4$ exhibited a lower affinity to RegIIA compared with wild-type $\alpha 3\beta 4$ nAChR. *C*, representative superimposed ACh-evoked currents obtained in the absence (control, *black line*) and presence of 100 nM RegIIA at wild-type $\alpha 3\beta 2$ (*red*), loop D mutant $\alpha 3\beta 2$ [T59K] (*green*), and loop E mutant $\alpha 3\beta 2$ [S113R] (*blue*). *D*, representative superimposed ACh-evoked currents obtained in the absence (control, *black line*) and presence of 100 nM RegIIA at wild-type (*red*) and mutant $\alpha 3\beta 4$ nAChRs (*green* and *blue*, respectively). *E*, bar graph summarizing the IC_{50} values with 95% CI obtained from concentration-response curves for RegIIA at wild-type $\alpha 3\beta 2$, $\alpha 3\beta 4$, and the mutants and chimeric subtypes analyzed. A gain in sensitivity was observed at mutants $\alpha 3\beta 2$ [T59K] and $\alpha 3\beta 2$ [S113R], whereas the most prominent reductions in sensitivity can be mapped to the opposite mutations $\alpha 3\beta 4$ [K59T] and $\alpha 3\beta 4$ [R113S]. *Dotted squares* indicate the agonist binding loops in which the respective mutants are located. All data points represent mean \pm 95% CI. The IC_{50} , 95% CI, and Hill slope (n_H) values are summarized in Table 1.

TABLE 1

RegIIA inhibition of $\alpha 3$ -containing nAChR subtypes and subtype mutants

IC_{50} values (nM) with 95% CI. Hill slope (n_H) was obtained from concentration-response curves for RegIIA at wild-type and mutant human $\alpha 3\beta 2$ and $\alpha 3\beta 4$ nAChR subtypes. Human $\alpha 3\beta 2$ nAChR mutations $\alpha 3\beta 2$ [T59K] and $\alpha 3\beta 2$ [S113R] notably decrease the IC_{50} of RegIIA towards the human $\alpha 3\beta 4$ subtype value, whereas the opposite human $\alpha 3\beta 4$ [K59T] and $\alpha 3\beta 4$ [R113S] result in lower potency of RegIIA and significantly increased IC_{50} values. Data from wild-type nAChRs and the mutants mentioned are highlighted in bold font. All data represent mean of $n = 3-9$ experiments.

nAChR/mutant	IC_{50}	95% CI	n_H
		<i>nM</i>	
$\alpha 3\beta 2$	132.4	109.7–159.7	1.5
$\alpha 3\beta 4$	45.6	31.5–65.9	0.9
$\alpha 3\beta 2$ [1–63 $\beta 4$]	159.2	113.0–224.4	1.4
$\alpha 3\beta 2$ [70–86 $\beta 4$]	1268.1	935.7–1718.5	0.9
$\alpha 3\beta 2$[T59K]	49.4	33.4–73.1	1.2
$\alpha 3\beta 4$[K59T]	2795.1	2346.6–3329.3	1.4
$\alpha 3\beta 4$ [113–115 $\beta 2$]	1056.5	924.9–1206.9	1.1
$\alpha 3\beta 2$[S113R]	23.7	19.0–29.4	1.5
$\alpha 3\beta 4$[R113S]	805.3	554.7–1169.0	0.9
$\alpha 3\beta 4$ [S114Y]	133.1	105.0–168.6	1.2
$\alpha 3\beta 4$ [N115D]	275.0	221.3–341.5	1.6
$\alpha 3\beta 4$ [M163K+T164S]	138.6	113.2–169.8	2.3
$\alpha 3\beta 4$ [P165E]	217.9	172.9–274.6	1.2
$\alpha 3\beta 4$ [T166V+M169L]	130.2	100.1–169.5	1.9

siderable shift of the concentration-response curve to the left compared with wild-type $\alpha 3\beta 2$ with an IC_{50} of 23.7 nM (95% CI 19.0–29.4 nM; $n_H = 1.5$) (Fig. 2, *A*, *C*, and *E*, and Table 1),

further confirming the key role of residue 113 in loop E in determining sensitivity toward RegIIA.

To probe the contribution of loop F to the sensitivity difference of RegIIA at human $\alpha 3\beta 4$ and $\alpha 3\beta 2$ we engineered three h $\beta 4$ receptor mutants: $\alpha 3\beta 4$ [M163K+T164S], $\alpha 3\beta 4$ [P165E], and $\alpha 3\beta 4$ [T166V+M169L]. When tested in oocytes, none of these three mutants revealed a notable loss of sensitivity to RegIIA (Fig. 2*E*, Table 1) indicating loop F does not play a key role in determining RegIIA sensitivity. Taken together, the mutational analysis revealed the non-conserved residue in loop D at position 59 (Lys in h $\beta 4$, Thr in h $\beta 2$) and residue Arg¹¹³ in h $\beta 4$ (Ser in h $\beta 2$) of loop E as the two major determinants for the subtype selectivity of RegIIA at human nAChRs.

Non-conserved Loop D and E Residues Differentially Affect the ACh Half-maximal Excitatory Concentrations (EC_{50}) at Human $\alpha 3\beta 4$ and $\alpha 3\beta 2$ nAChRs—We observed an ~ 18 -fold difference in the EC_{50} for ACh at human $\alpha 3\beta 4$ and $\alpha 3\beta 2$ nAChRs. The EC_{50} obtained at $\alpha 3\beta 2$ was 15.6 μM (95% CI 10.9–22.2 μM ; $n = 6$), whereas an EC_{50} of 287.6 μM (95% CI 274.1–301.8 μM , $n = 8$) was observed at $\alpha 3\beta 4$ (Table 2 and supplemental Fig. S1). In addition, the approximate EC_{50} values of the mutants at positions 59 and 113 as well as other $\alpha 3\beta 4$

Key Determinants for α -Conotoxin Interaction with $\alpha 3\beta 4$ nAChRs

TABLE 2

ACh EC_{50} values for wild-type and mutant human $\alpha 3\beta 2$ and $\alpha 3\beta 4$ nAChRs

EC_{50} values (μM) with 95% CI. The Hill slope (n_H) was obtained from concentration–response curves for ACh at wild-type and mutant human $\alpha 3\beta 2$ and $\alpha 3\beta 4$ nAChR subtypes. ACh concentrations from 0.01 μM to 10 mM (the highest applicable concentration) were tested. All data represent mean of $n = 4–8$ experiments.

nAChR/mutant	EC_{50}	95% CI	n_H
		μM	
h $\alpha 3\beta 2$	15.6	10.9–22.2	0.7
h $\alpha 3\beta 4$	287.6	274.1–301.8	2.3
h $\alpha 3\beta 2$ [T59K]	14.3	12.5–16.5	1.0
h $\alpha 3\beta 4$ [K59T]	29.8	27.9–31.9	1.1
h $\alpha 3\beta 2$ [S113R]	6.0	5.0–7.2	0.9
h $\alpha 3\beta 4$ [R113S]	42.9	36.4–50.5	1.1
h $\alpha 3\beta 4$ [S114Y]	65.1	56.3–75.3	1.1
h $\alpha 3\beta 4$ [N115D]	140.8	123.8–160.0	1.0
h $\alpha 3\beta 4$ [M163K+T164S]	63.1	58.6–67.9	1.0
h $\alpha 3\beta 4$ [P165E]	43.9	38.6–50.0	1.2
h $\alpha 3\beta 4$ [T166V+M169L]	17.5	15.8–19.4	1.1

mutants were determined. Mutant h $\alpha 3\beta 2$ [T59K] had a similar ACh EC_{50} to the wild-type h $\alpha 3\beta 2$ (14.3 μM , 95% CI 12.5–16.5 μM , $n = 4$). In contrast the opposite mutation h $\alpha 3\beta 4$ [K59T] exhibited a considerably lower EC_{50} than wild-type h $\alpha 3\beta 4$ (29.8 μM , 95% CI 27.9–31.9 μM , $n = 4$). Mutant h $\alpha 3\beta 4$ [R113S] also resulted in a considerably lower than wild-type h $\alpha 3\beta 4$ EC_{50} value of 42.9 μM (95% CI 36.4–50.5 μM , $n = 4$). Surprisingly, however, mutant h $\alpha 3\beta 2$ [S113R] revealed an EC_{50} in the low micromolar range (6.0 μM , 95% CI 5.0–7.2 μM , $n = 5$), which is comparable with wild-type h $\alpha 3\beta 2$ (Table 2 and supplemental Fig. S1). Overall, both $\beta 2$ mutants retained the high affinity to ACh as seen in wild-type h $\alpha 3\beta 2$, whereas the opposite mutations in h $\alpha 3\beta 4$ (h $\alpha 3\beta 4$ [K59T] and h $\alpha 3\beta 4$ [R113S]) considerably increased the affinity for ACh compared with h $\alpha 3\beta 4$. Other h $\alpha 3\beta 4$ loop E and F mutants that exhibited a less pronounced increase in RegIIA IC_{50} also had lower than wild-type ACh EC_{50} (Table 2). This is not unexpected given that these mutations are within the agonist binding loops.

The Non-conserved Residues h $\beta 4$ -Lys⁵⁹ and h $\beta 4$ -Arg¹¹³ Are the Key Determinants for Recovery from Block by RegIIA—Human $\alpha 3\beta 4$ and $\alpha 3\beta 2$ nAChRs not only exhibited a difference in sensitivity to α -conotoxin RegIIA with considerably different IC_{50} values, but also differences in the time required to recover from block. By repeatedly applying ACh under constant perfusion after incubation with the peptide, we measured the time required for the ACh-evoked current amplitude to recover.

Interestingly, RegIIA revealed notably different wash-off kinetics at h $\alpha 3\beta 2$ compared with h $\alpha 3\beta 4$. At h $\alpha 3\beta 2$, 95% recovery from block by RegIIA was reached within a minute (Fig. 3, A and B, and Table 3). In contrast, 95% wash-off at h $\alpha 3\beta 4$ took ~13–14 min (Fig. 4, A and B, and Table 3). A similar difference was observed at rat nAChRs, with faster recovery for r $\alpha 3\beta 2$ (1–2 min) compared with a considerably slower rate for r $\alpha 3\beta 4$ (16–18 min) (Table 3).

We next tested if any of the mutations that affected the IC_{50} of RegIIA also had an impact on the rate of recovery from block. Mutant h $\alpha 3\beta 4$ [K59T] exhibited a rapid off-rate, similar to that observed for wild-type h $\alpha 3\beta 2$ (95% recovery in <1 min) (Fig. 4, A and C). Conversely, peptide washout at the opposite mutant h $\alpha 3\beta 2$ [T59K] was very slow with less than 50% washout achieved after 45 min of perfusion (Fig. 3, A and C, and Table 3).

In rat $\beta 2$ and $\beta 4$ subunits, the residue at position 59 is homologous with human (Thr in $\beta 2$ and Lys in $\beta 4$). A similar slowing in wash-off was observed with rat $\alpha 3\beta 2$ [T59K] compared with wild-type r $\alpha 3\beta 2$ although less severe (Table 3), confirming the key role of residue 59 in loop D for the binding affinity of RegIIA.

Residue 113 mutants (h $\alpha 3\beta 2$ [S113R] and h $\alpha 3\beta 4$ [R113S]) also exhibited considerably different wash-off rates compared with the respective wild-type receptors. Peptide wash-off at h $\alpha 3\beta 4$ [R113S] was very fast, similar to the h $\alpha 3\beta 2$ subtype (95% wash-off in <1 min), whereas at h $\alpha 3\beta 2$ [S113R] it was almost as slow as at the opposite wild-type h $\alpha 3\beta 4$ nAChR (95% wash-off in 9–13 min) (Fig. 3A, D; Fig. 4, A and D, and Table 3).

In addition, mutants h $\alpha 3\beta 4$ [P165E] and h $\alpha 3\beta 4$ [T166V+M169L], which also showed a considerable higher affinity to ACh than wild-type, were tested for their RegIIA off-rate. Both mutants exhibited a faster off-rate compared with wild-type, which is consistent with the higher IC_{50} and lower EC_{50} observed. However, the wash-off was not as fast as observed for h $\alpha 3\beta 4$ [K59T] or h $\alpha 3\beta 4$ [R113S], placing these mutants between h $\alpha 3\beta 2$ and h $\alpha 3\beta 4$ with respect to their off-rates (Table 3).

In summary, these findings show that the residues at positions 59 and 113 not only affect the sensitivity of each receptor subtype to RegIIA, but they are also key determinants for the differences observed in wash-off kinetics. Other residues in the agonist binding loops also affected the affinities for ACh and the peptide, but to a lesser extent than the aforementioned positions and they appear to play an auxiliary role for peptide binding.

Molecular Modeling Shows the Interacting Mechanisms between the Non-conserved Residues of Human $\alpha 3\beta 4$ and $\alpha 3\beta 2$ nAChRs and RegIIA—The interactions between RegIIA and the binding sites of h $\alpha 3\beta 2$ and h $\alpha 3\beta 4$ nAChRs are very similar, with most of the residues in the first loop forming contacts with the (+) subunit and most of the second loop residues interacting with the (–) subunit.

The non-conserved residues at positions 59, 111, 113, and 119 of the β subunits form direct interactions with residues Asn⁹, Val¹⁰, and Asn¹¹ of the second loop of RegIIA (Fig. 5 and Table 4). The residues at positions 111 and 119 of h $\beta 2$ and h $\beta 4$ are hydrophobic and possess similar biophysical properties thus, explaining their minor effects on the sensitivity of RegIIA to h $\alpha 3\beta 2$ and h $\alpha 3\beta 4$ nAChRs.

In contrast, biophysical differences for the side chains at positions 59 and 113 are substantial, with two long and positively charged side chains contributed by residues Lys⁵⁹ and Arg¹¹³ at h $\beta 4$, whereas at the h $\beta 2$ subunit two neutral and short side chains from residues Thr⁵⁹ and Ser¹¹³ are present (Fig. 5, C–F). In our MD simulations, the side chains of $\beta 4$ -Lys⁵⁹ and Arg¹¹³ formed hydrogen bonds with backbone atoms of Asn⁹ and Cys¹⁶ (for $\beta 4$ -K59), and Asn¹¹ (for $\beta 4$ Arg¹¹³) in the loop 2 of RegIIA, whereas the side chains of $\beta 2$ Thr⁵⁹ and $\beta 2$ Ser¹¹³ are not long enough to form such interactions with the corresponding residues of RegIIA (Figs. 5, C–F, and 6). Residue $\beta 2$ Ser¹¹³ is instead forming hydrogen bonds with the adjacent $\beta 2$ Asp¹¹⁵ and $\beta 2$ Ser¹¹⁷ residues, which stabilize it in an orientation facing away from the peptide binding site (Fig. 5E).

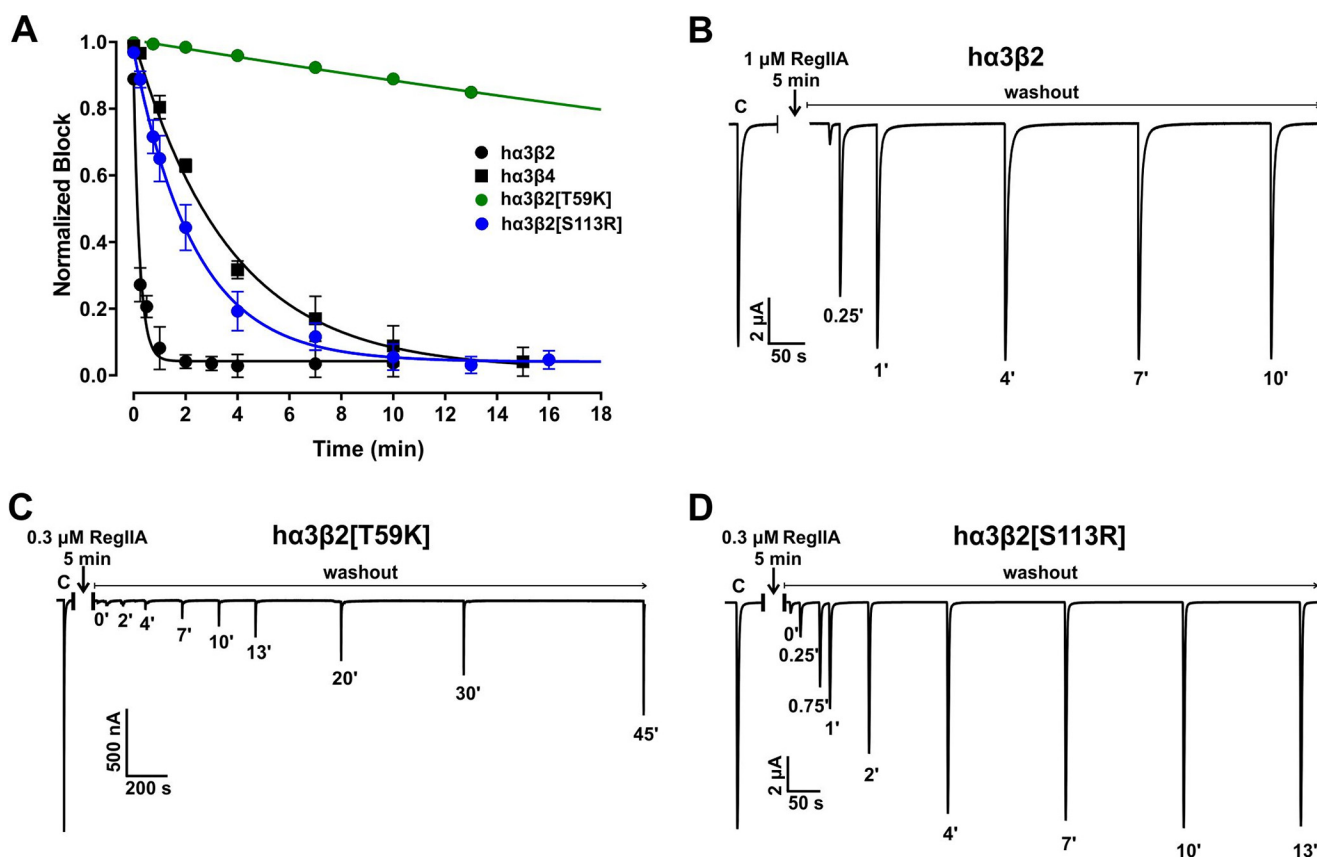


FIGURE 3. Wild-type human $\alpha 3\beta 2$ and $\alpha 3\beta 4$ nAChRs display different wash-off kinetics from block by α -conotoxin RegIIA. Single residue $\beta 2$ mutations in $\alpha 3\beta 2$ are sufficient to switch off-rates to those of the opposite subtype. *A*, graph summarizing the wash-off kinetics data. The $\alpha 3\beta 2$ nAChR subtype (*black circles*) exhibits fast recovery of ACh-evoked currents from block by RegIIA, with full recovery achieved in less than 2 min. In contrast, at $\alpha 3\beta 4$ (*black squares*) currents recovered from RegIIA block after a 13–14-min washout. When loop D residue 59 in $\beta 2$ was replaced with the respective residue of $\beta 4$ (mutant $\alpha 3\beta 2$ [T59K], *green circles*) the off-rate of RegIIA was dramatically slowed. A similar replacement of loop E residue 113 in $\beta 2$ (mutant $\alpha 3\beta 2$ [S113R], *blue circles*) slowed the recovery rate similar to $\alpha 3\beta 4$. Representative ACh-evoked currents of $\alpha 3\beta 2$ (*B*), $\alpha 3\beta 2$ [T59K] (*C*), and $\alpha 3\beta 2$ [S113R] (*D*) illustrate the recovery from the RegIIA block differs between wild-type and the two mutant $\alpha 3\beta 2$ nAChR subtypes. Numbers at the respective ACh-evoked current peaks indicate the duration (in min) of washout and C indicates a representative control ACh application before incubation with the peptide. Oocytes were incubated with RegIIA for 5 min followed by repetitive application of ACh under continuous perfusion with ND96 solution. Approximate EC_{50} values for ACh and RegIIA concentrations giving major to full block of ACh-evoked currents under these conditions were used for each subtype tested. All data points in *A* represent mean \pm 95% CI, $n = 3$ –7. The times required to reach 95% recovery from block are summarized in Table 3.

TABLE 3
Recovery time from block by α -conotoxin RegIIA

nAChR/mutant	t_{95}^a
	min
$\alpha 3\beta 2$	1
$\alpha 3\beta 4$	13–14
$\alpha 3\beta 2$	1–2
$\alpha 3\beta 4$	16–18
$\alpha 3\beta 2$ [T59K]	>45
$\alpha 3\beta 4$ [K59T]	<1
$\alpha 3\beta 2$ [T59K]	>45
$\alpha 3\beta 4$ [R113S]	<1
$\alpha 3\beta 2$ [S113R]	9–3
$\alpha 3\beta 4$ [P165E]	3–5
$\alpha 3\beta 4$ [T166V+M169L]	5–7

^a Time to reach 95% recovery after continuous peptide washout.

MD simulation of a RegIIA-bound mutant $\alpha 3\beta 4$ [K59T] model revealed that the $\beta 4$ Lys⁵⁹ to Thr mutation removed the hydrogen bond between the RegIIA amide terminus and the position 59 side chain, thereby significantly decreasing the affinity of RegIIA compared with wild-type $\alpha 3\beta 4$. Although the conformation of residues near position 59 was not significantly perturbed by the mutation, the non-conserved residues near the binding site have an unfavorable effect for RegIIA

binding at $\alpha 3\beta 4$ [K59T]. MD simulation indicates the opposite mutant $\alpha 3\beta 2$ [T59K] exhibits higher affinity to RegIIA compared with wild-type $\alpha 3\beta 2$, because RegIIA is stabilized by additional hydrogen bonds formed between $\beta 2$ Lys⁵⁹, Asp¹⁷⁰, and the amide group of RegIIA. However, this mutation also introduced significant local conformation changes of the $\beta 2$ binding interface, thereby decreasing contacts between RegIIA and the $\beta 2$ residue side chains Phe¹¹⁹ and Lys¹⁶³. These effects counteract the affinity gain resulting from introduction of Lys⁵⁹, and are likely responsible for the overall only mildly increased RegIIA activity at mutant $\alpha 3\beta 2$ [T59K].

Computational studies on a RegIIA-bound mutant $\alpha 3\beta 2$ -[S113R] model revealed that residue $\beta 2$ Arg¹¹³ forms salt bridges with Asp¹¹⁵, similar to Ser¹¹³ in wild-type $\alpha 3\beta 2$ (see Fig. 5E), instead of directly interacting with Asn¹¹ of RegIIA. Interestingly we observed that the backbone oxygen atom of Asn¹¹ forms a hydrogen bond with Lys⁷⁹ rather than Arg¹¹³ due to the relocation of the latter. Thus, our MD simulation indicates that Arg¹¹³ of $\alpha 3\beta 2$ [S113R] strengthens the binding of RegIIA through stabilization of the local conformation

Key Determinants for α -Conotoxin Interaction with $\alpha 3\beta 4$ nAChRs

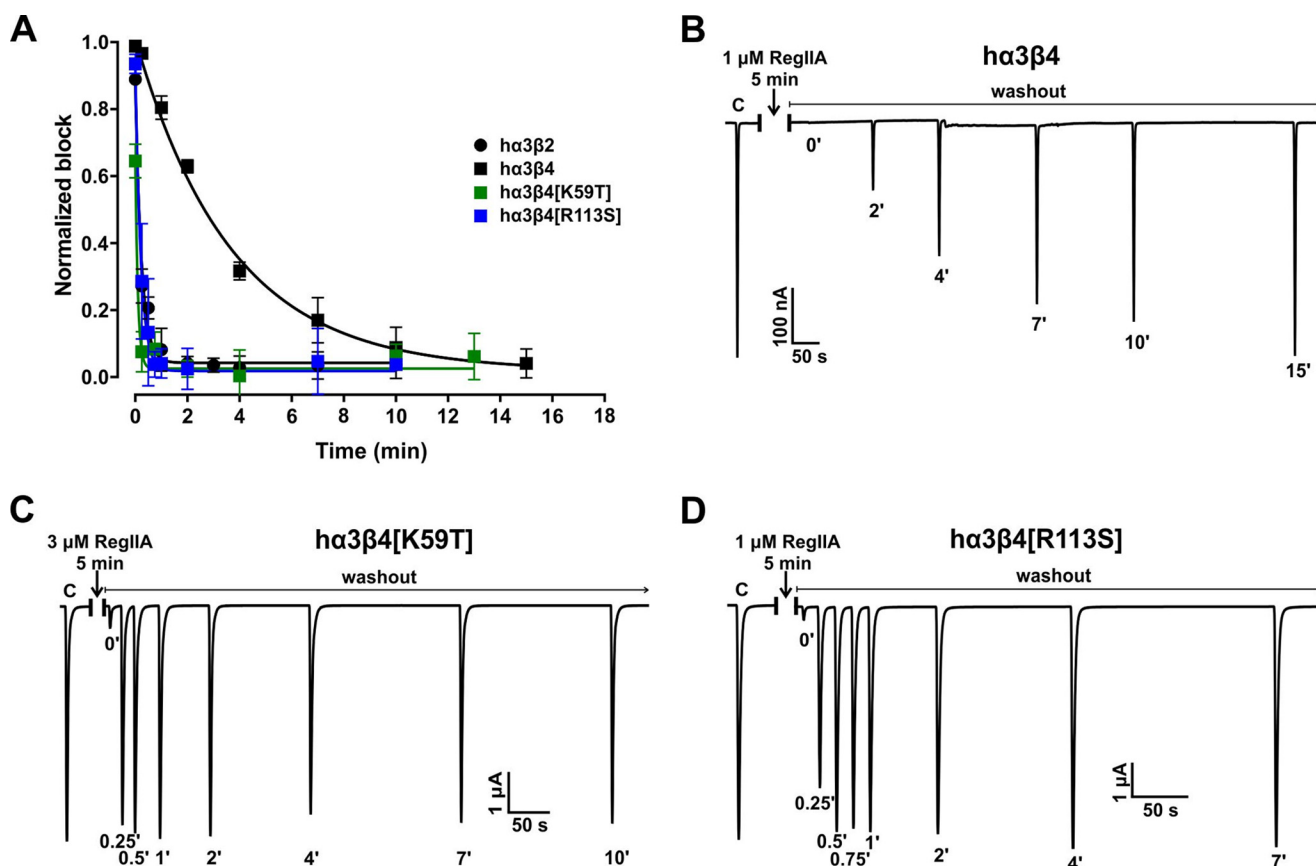


FIGURE 4. Single residue $\beta 4$ mutations in $\alpha 3\beta 4$ increase the α -conotoxin off-rate of RegIIA to values similar to wild-type $\alpha 3\beta 2$. *A*, graph summarizing the wash-off kinetics data. Wild-type $\alpha 3\beta 2$ and $\alpha 3\beta 4$ data are the same as described in the legend to Fig. 3 and shown for comparison. Replacing loop D residue 59 in $\beta 4$ with the respective residue in $\beta 2$ (mutant $\alpha 3\beta 4$ [K59T], green squares) is sufficient to shift the off-rate to the opposite subtype. A mutant in which residue Arg¹¹³ of $\beta 4$ is replaced with Ser, as in $\beta 2$, ($\alpha 3\beta 4$ [R113S], blue squares) similarly exhibits a fast wash-off rate resembling $\alpha 3\beta 2$. Representative ACh-evoked currents of $\alpha 3\beta 4$ (*B*), $\alpha 3\beta 4$ [K59T] (*C*), and $\alpha 3\beta 4$ [R113S] (*D*) illustrate the recovery from the RegIIA block differs between wild-type and the two mutant $\alpha 3\beta 4$ nAChR subtypes. Experimental conditions were the same as those described in the legend to Fig. 3. All data points in *A* represent mean \pm 95% CI, $n = 3-7$. The times required to reach 95% recovery from block are summarized in Table 3.

near the binding site, thereby making mutant $\alpha 3\beta 2$ [S113R] even more RegIIA-sensitive than wild-type $\alpha 3\beta 4$.

Furthermore, we computationally analyzed RegIIA binding to other $\alpha 3\beta 4$ mutants that exhibited a major loss in RegIIA activity. The loop F mutation in $\alpha 3\beta 4$ [P165E] created a local conformation change to the entire agonist binding loop F. We identified that the angle defined by the C α atoms at 164, 165, and 166 positions became significantly larger (right shift). We conclude that this local conformation change might affect RegIIA binding to the $\alpha 3\beta 4$ [P165E], but that Pro¹⁶⁵, or its neighboring residues, are not determinants for RegIIA binding in the wild-type nAChR subtypes.

The $\alpha 3\beta 4$ [N115D] mutant, which also showed significantly decreased sensitivity to RegIIA compared with wild-type $\alpha 3\beta 4$, was analyzed further. Based on MD simulation data we suggest $\beta 4$ Asp¹¹⁵ might compete with residues of RegIIA to interact with Arg¹¹³. Similar to the interaction of $\beta 2$ Ser¹¹³ in $\alpha 3\beta 2$ (Fig. 5, *A* and *E*) residue $\beta 4$ Arg¹¹³ forms salt bridges with Asp¹¹⁵ rather than with residues of RegIIA in $\alpha 3\beta 4$ [N115D]. This explains the mutational effects of [N115D] to the binding affinity of RegIIA. Although the opposite mutation in $\beta 2$ (mutant $\alpha 3\beta 2$ [D115N]) would weaken the H-bond with Ser¹¹³, the overall effect would not be a reciprocal

gain in RegIIA affinity, because the Ser¹¹³ residue is too short to directly interact with RegIIA.

Overall, our computationally directed mutational studies suggest that mutants $\alpha 3\beta 4$ [P165E] and $\alpha 3\beta 4$ [N115D] negatively affect RegIIA binding through mildly altering the local conformation of either the backbone or the side chains of the residues near the binding site. But those non-conserved positions do not appear to determine the difference in RegIIA sensitivity between wild-type $\alpha 3\beta 2$ and $\alpha 3\beta 4$ to a similar extent as do residues 59 and 113.

The non-conserved β subunit residues at positions 34, 36, 71, 78, 79, 114, 115, 163, 164, 165, and 166 are far from the binding site, and their side chains merely form weak interactions with RegIIA. Their effects to the binding affinity of RegIIA are mostly indirect, by forming hydrogen bonds with neighboring residues to affect the local conformation of the binding site (Fig. 5).

Taken together, MD simulations indicate the non-conserved residues 59 and 113 of the (–) interface are crucial for the observed differences in RegIIA activity, due to their considerably different mode of interaction with the peptide. In $\alpha 3\beta 4$ nAChRs, hydrogen bonds are formed between the respective residues and RegIIA loop 2, but they are absent in $\alpha 3\beta 2$.

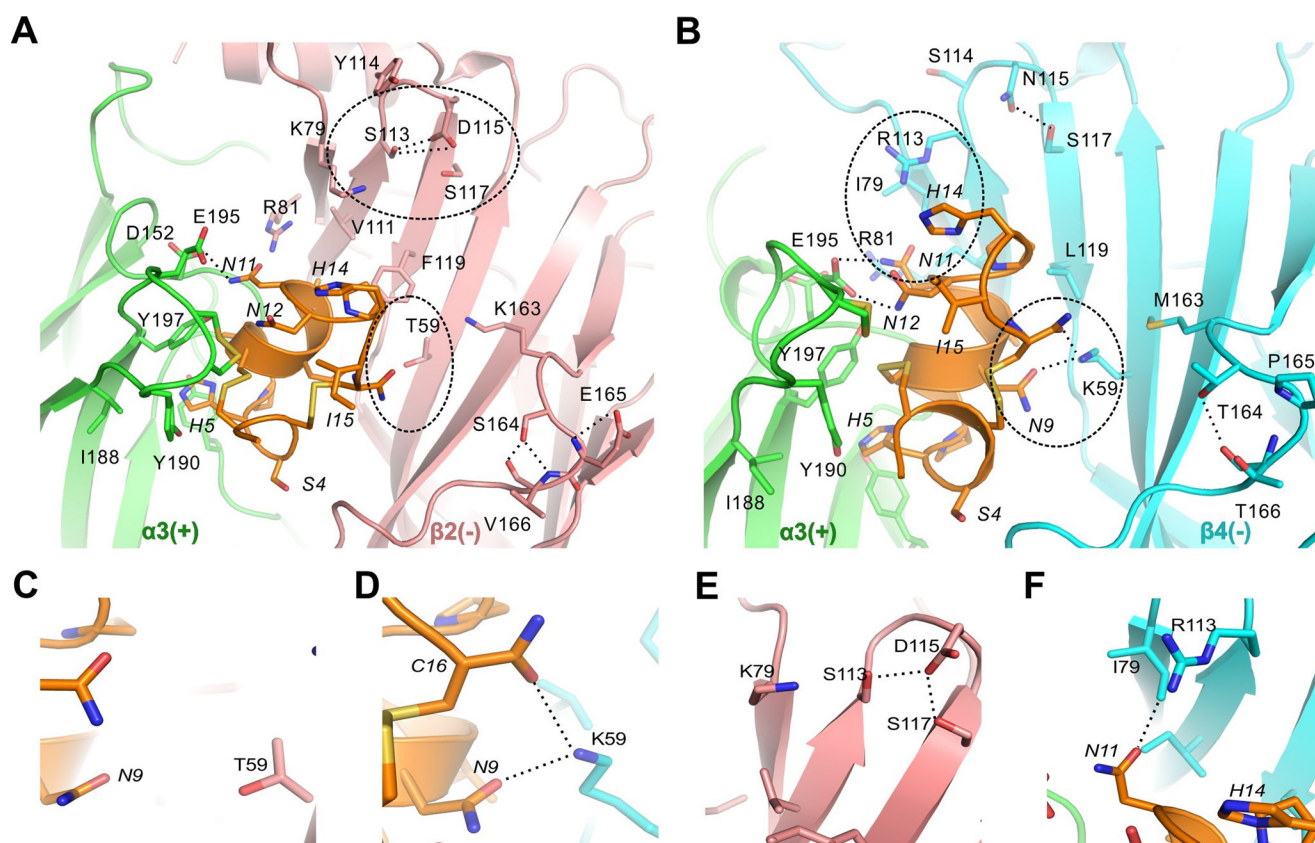


FIGURE 5. Molecular docking models illustrate the binding modes of RegIIA to $\alpha 3\beta 2$ (A) and $\alpha 3\beta 4$ (B), respectively. Several hydrogen bonds (dashed lines) are formed between pairwise interacting residues of different loops or β -sheets, thereby affecting their local conformation or dynamics, which in turn affects the binding of RegIIA. Note that hydrogen bonds are formed by several non-conserved $\beta 2$ -subunit (Ser¹¹³, Asp¹¹⁵, Ser¹⁶⁴, Glu¹⁶⁵) and $\beta 4$ -subunit residues (Asn¹¹⁵, Thr¹⁶⁴). The $\alpha 3(+)$ interface is shown in green, $\beta 2(-)$ in pink, $\beta 4(-)$ in cyan, and RegIIA in orange. Residues near the agonist binding site that affect RegIIA binding are shown as licorice models. Residues from the receptor and RegIIA are labeled using normal and *italic fonts*, respectively. Key interaction sites responsible for the differences in α -conotoxin RegIIA binding are highlighted with dashed circles. C–F, magnification of the key sites in $\alpha 3\beta 2$ (C and E) and $\alpha 3\beta 4$ (D and F) highlighted with circles in A and B.

TABLE 4
Contacts of α -conotoxin RegIIA with $\alpha 3\beta 2$ and $\alpha 3\beta 4$ nAChR, respectively

Contacts between nAChR and RegIIA are defined as van der Waals interactions if the distance between heavy atoms of RegIIA and nAChR is between 2.6 and 4 Å. Residues of the nAChR forming hydrogen bonds with RegIIA are underlined. Residues that are non-conserved between $\alpha 3\beta 2$ and $\alpha 3\beta 4$ nAChRs, as well as RegIIA residues making contact with them are shown in bold.

Residue ^a	$\alpha 3\beta 2$ nAChR		$\alpha 3\beta 4$ nAChR	
	+ ^b	- ^c	+ ^b	- ^c
Ser ⁴		Asp ¹⁷¹		Asp ¹⁷¹
His ⁵	Tyr ⁹³ , Tyr ¹⁹⁰ , Tyr ¹⁹⁷		Tyr ⁹³ , Tyr ¹⁹⁰ , Tyr ¹⁹⁷	
Pro ⁶	Tyr ⁹³ , Trp ¹⁴⁹	Trp ⁵⁷	Tyr ⁹³ , Trp ¹⁴⁹	Leu ¹²¹
Ala ⁷	Ser ¹⁵⁰ , Tyr ¹⁹⁷		Ser ¹⁵⁰ , Tyr ¹⁹⁷	
Asn ⁹		Trp ⁵⁷ , Thr ⁵⁹ , Phe ¹¹⁹ , Leu ¹²¹		Lys⁵⁹ , Leu ¹²¹
Val ¹⁰	Ser ¹⁵⁰	Arg ⁸¹ , Val ¹¹¹ , Phe ¹¹⁹ , Leu ¹²¹	Ser ¹⁵⁰	Arg ⁸¹ , Ile ¹¹¹ , Leu ¹¹⁹ , Leu ¹²¹
Asn ¹¹	Asp ¹⁵² , Glu ¹⁹⁵ , Tyr ¹⁹⁷	Lys ⁷⁹ , Arg⁸¹ , Val ¹¹¹	Asp ¹⁵² , Glu¹⁹⁵ , Tyr ¹⁹⁷	Ile ⁷⁹ , Arg ⁸¹ , Ile ¹¹¹
Asn ¹²	Cys ¹⁹² , Cys ¹⁹³ , Glu ¹⁹⁵		Cys ¹⁹² , Cys ¹⁹³ , Glu¹⁹⁵	
His ¹⁴	Cys ¹⁹² , Cys ¹⁹³		Cys ¹⁹² , Cys ¹⁹³	
Ile ¹⁵	Cys ¹⁹² , Cys ¹⁹³		Cys ¹⁹² , Cys ¹⁹³	

^a Only RegIIA residues making direct contact with the nAChRs are listed.

^b Residues of the principal side of the binding site.

^c Residues of the complementary side of the binding site.

The $\alpha 3\beta 4$ nAChR-selective α -Conotoxin AuIB Is Less Active at the Homologous Human nAChR Subtype—The 4/6 α -conotoxin AuIB has been shown to selectively inhibit the rat $\alpha 3\beta 4$ nAChR with an IC₅₀ of 0.75 μ M (13). Activity at human nAChRs has not yet been reported. Therefore, we determined the activity of AuIB at wild-type $\alpha 3\beta 2$, $\alpha 3\beta 4$, and relevant β subunit mutants. Interestingly, 1 μ M AuIB was inactive at $\alpha 3\beta 4$ and exhibited a minor block of ACh-evoked currents (10.7 \pm 3.2%, n = 9) at $\alpha 3\beta 2$ nAChR. The nAChR β subunit mutants that

distinctly affected the RegIIA activity profile (positions 59 and 113) were all relatively insensitive to 1 μ M AuIB (Fig. 7A). Concentration-response analyses with wild-type $\alpha 3\beta 2$ and $\alpha 3\beta 4$ nAChRs confirmed the low sensitivity of both nAChR subtypes to AuIB. α -Conotoxin AuIB at 30 μ M, reduced ACh-evoked currents mediated by $\alpha 3\beta 2$ to 75.9 \pm 3.7% of control (n = 10) and $\alpha 3\beta 4$ -mediated currents to only 79.6 \pm 4.0% (n = 10) of control, indicating that the IC₅₀ is considerably higher than 30 μ M at either nAChR subtype (Fig. 7B).

Key Determinants for α -Conotoxin Interaction with $\alpha 3\beta 4$ nAChRs

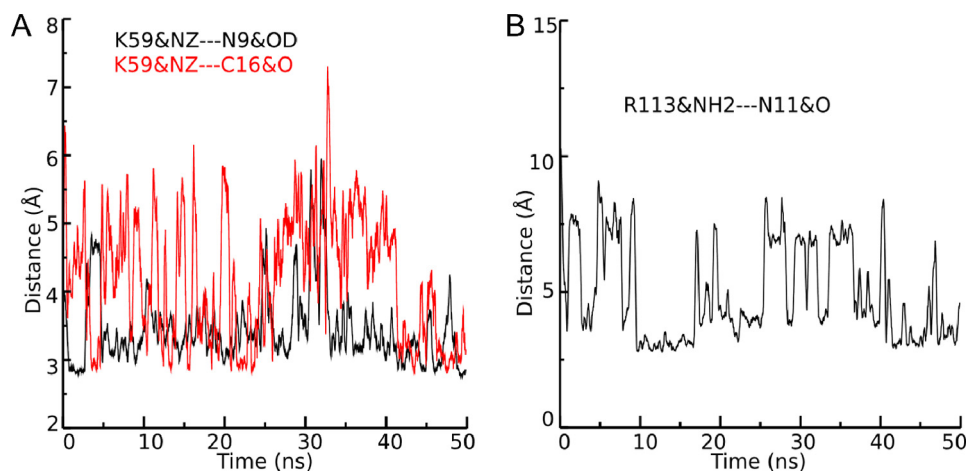


FIGURE 6. MD simulations of 50-ns duration show the evolution of distances between $\beta 4$ Lys⁵⁹ heavy atoms and RegIIA residues Asn⁹ as well as Cys¹⁶ (A), and between $\beta 4$ Arg¹¹³ and RegIIA Asn¹¹ (B), respectively. The strengthening of the hydrogen bond is reversely proportional to the distance between hydrogen bond donor and hydrogen bond acceptor. When the distance between the heavy atoms is more than 3.5 Å, the strength of the hydrogen bond is believed to be weak. Red and black colors are used to discriminate the two simulations performed with $\beta 4$ Lys⁵⁹. The labels *K59&NZ-N9&OD* and *K59&NZ-C16&O* designate the distance between the heavy atom NA of residue $\beta 4$ Lys⁵⁹ and the heavy atom OD of residue RegIIA Asn⁹, and the distance between heavy atom *K59&NZ* and the heavy atom O of residue RegIIA C16, respectively. The label *R113&NH2-N11&O* refers to the distance between the heavy atoms NH₂ of $\beta 4$ Arg¹¹³ and O of RegIIA Asn¹¹.

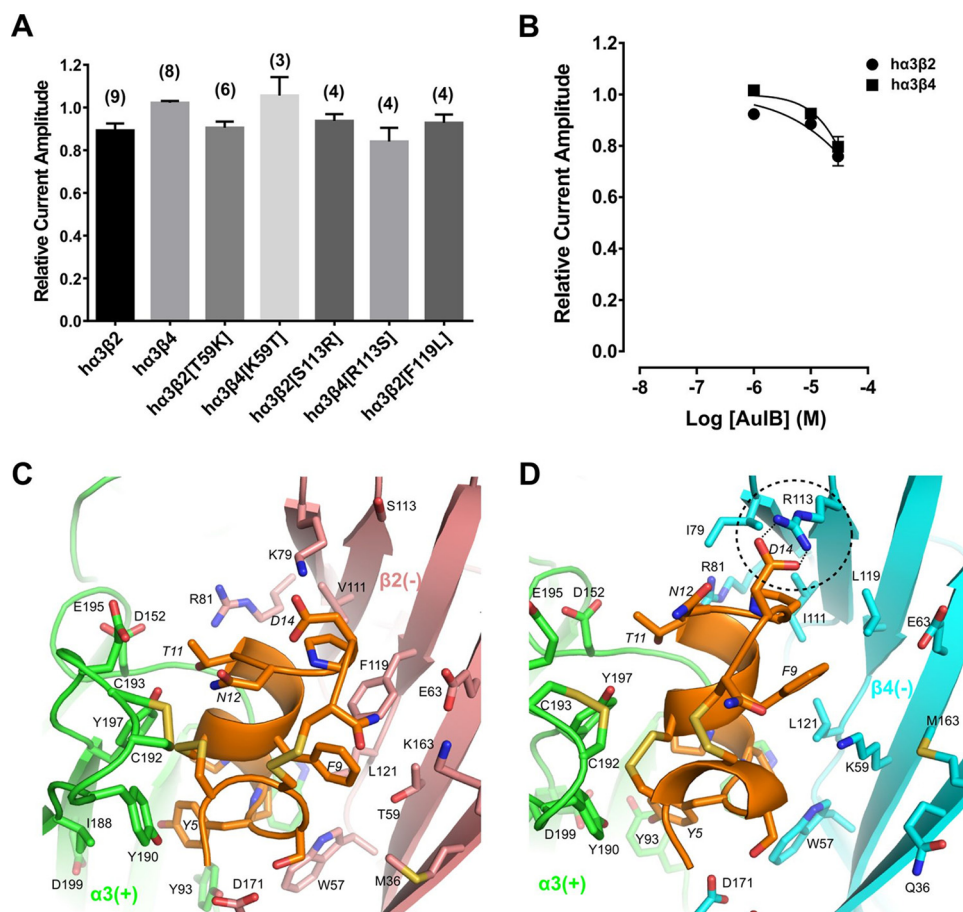


FIGURE 7. α -Conotoxin AulB has minor activity at human $\alpha 3\beta 2$ and $\alpha 3\beta 4$ nAChRs. A, bar graph representing potency of block by α -conotoxin AulB ($1 \mu\text{M}$) at wild-type and mutant human $\alpha 3\beta 2$ and $\alpha 3\beta 4$ nAChRs. Data represent mean \pm S.E., $n = 3-9$. B, concentration-response analysis of AulB at wild-type $\alpha 3\beta 2$ and $\alpha 3\beta 4$ nAChRs indicated the IC_{50} is considerably higher than $30 \mu\text{M}$ at both nAChR subtypes. AulB ($30 \mu\text{M}$) reduced ACh-evoked current amplitude mediated by $\alpha 3\beta 2$ to $75.9 \pm 3.7\%$ of control ($n = 10$) and $\alpha 3\beta 4$ currents to $79.6 \pm 4.0\%$ ($n = 10$), respectively. C and D, molecular dynamics simulation predicted binding modes of AulB to $\alpha 3\beta 2$ (C) and $\alpha 3\beta 4$ (D). Several hydrogen bonds are formed between pairwise interacting residues of different loops and between toxin and receptor, e.g. AulB Asp¹⁴ with $\beta 4$ Arg¹¹³ (dashed circle, hydrogen bonds as dotted lines). The $\alpha 3(+)$ interface is shown in green, $\beta 2(-)$ in pink, $\beta 4(-)$ in cyan, and AulB in orange. Non-conserved residues are shown as licorice models and labeled. Residues from the receptor and AulB are labeled using normal and italic fonts, respectively.

TABLE 5

Contacts of α -conotoxin AuIB with $\alpha 3\beta 2$ and $\alpha 3\beta 4$ nAChRs, respectively

Contacts between nAChR and AuIB are defined as van der Waals interactions if the distance between heavy atoms of AuIB and nAChR is between 2.6 and 4 Å. Residues of the nAChR forming hydrogen bonds with AuIB are underlined. Residues that are non-conserved between $\alpha 3\beta 2$ and $\alpha 3\beta 4$ nAChRs, as well as AuIB residues making contact with them are shown in bold.

Residue ^a	$\alpha 3\beta 2$ nAChR		$\alpha 3\beta 4$ nAChR	
	+ ^b	- ^c	+ ^b	- ^c
Ser ⁴		<u>Asp¹⁷¹</u>		<u>Asp¹⁷⁰</u>
Tyr ⁵	Tyr ⁹³ , Tyr ¹⁹⁰ , Asp ¹⁹⁹		Tyr ⁹³ , Tyr ¹⁹⁰ , Asp ¹⁹⁹	
Pro ⁶	Trp ¹⁴⁹	Trp ⁵⁷ , Leu ¹²¹	Trp ¹⁴⁹	Trp ⁵⁷ , Leu ¹²¹
Pro ⁷	Trp ¹⁴⁹ , Ser ¹⁵⁰ , Tyr ¹⁹⁷		Trp ¹⁴⁹ , Ser ¹⁵⁰ , Tyr ¹⁹⁷	
Phe⁹		Met³⁶, Trp⁵⁷, Thr⁵⁹, Phe¹¹⁹, Leu¹²¹		Lys⁵⁹, Glu⁶¹, Leu¹¹⁹, Leu¹²¹
Ala ¹⁰	Ser ¹⁵⁰	Arg ⁸¹ , Val ¹¹¹ , Phe ¹¹⁹	Ser ¹⁵⁰	Arg ⁸¹ , Ile ¹¹¹
Thr ¹¹	Asp ¹⁵² , Glu ¹⁹⁵ , Tyr ¹⁹⁷		Asp ¹⁵² , Glu ¹⁹⁵ , Tyr ¹⁹⁷	
Asn ¹²	Cys ¹⁹² , Cys ¹⁹³		Cys ¹⁹² , Cys ¹⁹³	
Pro ¹³		Lys ⁷⁹ , Val ¹¹¹ , Phe ¹¹⁹		Ile ¹¹¹ , Arg ¹¹³
Asp¹⁴		Lys ⁷⁹		Arg¹¹³

^a Only AuIB residues making direct contact with the nAChRs are listed.

^b Residues of the principal side of the binding site.

^c Residues of the complementary side of the binding site.

MD Simulation of α -Conotoxin AuIB Binding to $\alpha 3\beta 2$ and $\alpha 3\beta 4$ nAChRs, Compared with RegIIA, Indicates Other Key Interactions Affect Binding—In MD simulations, the $\alpha 4/6$ -conotoxin AuIB binds at the interface between the subunits and overlaps with the agonist binding sites of $\alpha 3\beta 2$ and $\alpha 3\beta 4$ nAChRs. Overall molecular interactions are relatively similar to those with RegIIA, as most of AuIB loop 1 residues form contacts with the (+)-subunit and most of the residues at loop 2 interact with residues at the (-)-subunit (Fig. 7, C and D, and Table 5). However, despite the general similarity of RegIIA and AuIB binding to $\alpha 3\beta 2$ and $\alpha 3\beta 4$ nAChRs, several side chains, including the non-conserved residues at positions 59 and 113 of both nAChR β subunits, formed remarkably different interactions with residues in loop 2 of AuIB (Fig. 7, C and D, and Table 5). These differences are a result of a significant shift in orientation between the two α -conotoxins. Direct comparison of the orientation of AuIB with RegIIA at the $\alpha 3(+)\beta 4(-)$ binding interface revealed that the shifted AuIB backbone places AuIB Phe⁹ far away from the $\beta 4$ Trp⁵⁷ side chain. Thereby no direct interaction between the aromatic ring of AuIB Phe⁹ and the tryptophan can occur. Instead AuIB Phe⁹ is in relatively close proximity to $\beta 4$ Leu¹¹⁹ and $\beta 4$ Lys⁵⁹ (Fig. 7D). It is likely the missing interaction between the AuIB Phe⁹ aromatic ring and $\beta 4$ Trp⁵⁷ is a significant factor for the lack of activity of AuIB at $\alpha 3\beta 4$, whereas for $\alpha 3\beta 2$ it is the insufficient stabilization of AuIB Phe⁹ by residue $\beta 2$ Thr⁵⁹.

Discussion

The $\alpha 3\beta 4$ nAChR subtype represents an important pharmacological target as it is involved in several pathophysiological disease conditions such as lung cancer, nicotine addiction, and drug abuse (3, 11, 12). To date, only a few α -conotoxins have been found to be active at the $\alpha 3\beta 4$ nAChR subtype and even fewer are highly specific for it. A problem complicating the task of developing specific $\alpha 3\beta 4$ receptor antagonists is that the ligand binding sites are structurally very similar among nAChR subtypes. However, the small structural differences between subtypes can cause profound pharmacological effects, as seen with the selectivity profiles of various α -conotoxins.

α -Conotoxin RegIIA has previously been shown to exhibit a species-specific difference in activity between human and rat

$\alpha 3\beta 2$ nAChRs, whereas this species difference was not observed at the $\alpha 3\beta 4$ nAChR subtype (17). Given that both the species and subtype selectivity profiles are well described for RegIIA, it represents a suitable probe to investigate pharmacological differences between $\alpha 3\beta 2$ and $\alpha 3\beta 4$ nAChR subtypes in detail.

RegIIA inhibits ACh-evoked currents competitively, like most α -conotoxins, indicating that its binding site overlaps with the agonist binding site (16). Sequence alignment between human $\beta 4$ and $\beta 2$ subunits shows their extracellular agonist binding regions are moderately conserved on the amino acid level between subtypes (70% homology, Fig. 1A). A mutational approach was chosen to elucidate how non-conserved structural elements account for functional differences in α -conotoxin binding.

Using receptor chimeras and point mutations to exchange individual residues to those of the opposite subtype, we identified two key residues (at positions 59 and 113, respectively) that crucially determine the selectivity of RegIIA. Exchange of $\beta 2$ Thr⁵⁹ to Lys increased RegIIA sensitivity similar to that observed at $\alpha 3\beta 4$, whereas the opposite exchange of $\beta 4$ Lys⁵⁹ to Thr resulted in a 61-fold loss of sensitivity compared with wild-type $\alpha 3\beta 4$. Furthermore, the rate of recovery from block by RegIIA differs considerably between $\alpha 3\beta 2$ and $\alpha 3\beta 4$. By exchanging the single residue at position 59 of the β subunit, we were able to swap the wash-off rate of subtypes.

Residue 113 on the β subunit has a similarly important role for RegIIA selectivity. Exchange of $\beta 2$ Ser¹¹³ to Arg made the mutant $\alpha 3\beta 2$ [S113R] receptor twice as sensitive to RegIIA than wild-type $\alpha 3\beta 4$. In contrast, the opposite mutation in $\beta 4$ made the mutant receptor ($\alpha 3\beta 4$ [R113S]) 18-fold less sensitive to RegIIA than wild-type $\alpha 3\beta 4$ and 34-fold less sensitive than mutant $\alpha 3\beta 2$ [S113R]. In addition, the single exchange of residue 113 between $\beta 2$ and $\beta 4$ was sufficient to switch the off-rate of RegIIA to that of the opposite subtype. To our knowledge, this is the first time Arg¹¹³ at the complementary agonist binding interface of nAChRs has been identified as a key determinant in antagonist binding. Computational modeling of RegIIA-bound mutant $\alpha 3\beta 2$ [S113R] provided insights about why this mutant is even more sensitive to RegIIA than $\alpha 3\beta 4$.

Key Determinants for α -Conotoxin Interaction with $\alpha 3\beta 4$ nAChRs

When Arg¹¹³ is introduced into the $\beta 2$ backbone it forms salt bridges with Asp¹¹⁵, similar to Ser¹¹³ in wild-type $\alpha 3\beta 2$ (see Fig. 5E). However, in the case of Arg¹¹³ this interaction also affects the conformations of residues near the binding site in such way that residue $\beta 2$ Lys⁷⁹ can form a hydrogen bond with the backbone oxygen atom of RegIIA Asn¹¹.

It is important to note that the ACh EC₅₀ at $\alpha 3\beta 2$ is ~18-fold lower than at $\alpha 3\beta 4$. As the aforementioned β -subunit residues are both within the agonist binding region at the interface between the α and β subunits, it can be assumed that point mutations would affect the EC₅₀ of ACh. Indeed mutant $\alpha 3\beta 4$ [R113S] exhibited ~7-fold lower EC₅₀ than wild-type $\alpha 3\beta 4$. However, unlike the IC₅₀ of RegIIA, there was no reciprocal effect on ACh EC₅₀ with the opposite mutant $\alpha 3\beta 2$ [S113R]. This mutant exhibited an even slightly lower EC₅₀ than $\alpha 3\beta 2$. Comparably, both $\alpha 3\beta 2$ [T59K] and $\alpha 3\beta 4$ [K59T] revealed an EC₅₀ similar to $\alpha 3\beta 2$ (Table 2). This discrepancy suggests the mutations affecting ACh EC₅₀ most likely impact the observed RegIIA potencies as well, as ACh competes with RegIIA for the agonist binding site. Mutants $\alpha 3\beta 2$ [T59K] and $\alpha 3\beta 2$ [S113R] are already more RegIIA sensitive than wild-type $\alpha 3\beta 2$, but it can be assumed they would be even more sensitive if their ACh EC₅₀ values were not considerably lower than the EC₅₀ seen with $\alpha 3\beta 4$.

Other $\alpha 3\beta 4$ mutants, primarily within loop F, also decreased RegIIA sensitivity and increased ACh affinity. Compared with the aforementioned $\alpha 3\beta 4$ [K59T] and $\alpha 3\beta 4$ [R113S] mutants, we observed a less extensive increase in RegIIA IC₅₀ and RegIIA wash-off kinetics. We hypothesize the other mutants play an auxiliary role for the subtype selectivity of RegIIA.

Residue 59 on the β subunits of rat nAChRs has previously been identified as a determinant for α -conotoxin LvIA potency and wash-off kinetics (31). α -Conotoxin LvIA exhibited a 17-fold higher selectivity for rat $\alpha 3\beta 2$ over $\alpha 3\beta 4$, and the rat $\alpha 3\beta 2$ [T59K] mutation further increased the activity of the peptide by ~10-fold. Furthermore, similar to our findings with RegIIA, mutant $\alpha 3\beta 2$ [T59K] considerably slowed the recovery from block by LvIA compared with wild-type $\alpha 3\beta 2$ (31).

In our MD simulations, the evolution of distances between key RegIIA and receptor residues over 50 ns revealed the involvement of the $\beta 4$ Lys⁵⁹ residue side chain in forming hydrogen bonds with main chain atoms of RegIIA Asn⁹ and Cys¹⁶ residues, and similarly, $\beta 4$ Arg¹¹³ formed a hydrogen bond with the Asn¹¹ in loop 2 of RegIIA (Figs. 5 and 6). Both RegIIA and LvIA have an asparagine at position 9. Therefore, it is likely the Asn⁹ residue interacts with $\beta 4$ Lys⁵⁹ via a direct hydrogen bond in both peptides. The increased potency and slower wash-off caused by the point mutation of $\beta 2$ Thr⁵⁹ to Lys could then be explained by gain of these additional hydrogen bonds, resulting in tighter binding of the peptide to the mutant nAChR. The side chain of $\beta 2$ Thr⁵⁹, however, is not long enough to form such interactions with the corresponding residues in loop 2 of RegIIA (Fig. 5C), which would explain the faster wash-off at wild-type $\alpha 3\beta 2$ and mutant $\alpha 3\beta 4$ [K59T], as well as lower potency of the peptide compared with $\alpha 3\beta 4$ and mutant $\alpha 3\beta 2$ [T59K], respectively.

Furthermore, MD simulation of RegIIA binding to mutant $\alpha 3\beta 4$ [K59T] revealed that the mutation also removed a H-bond between the RegIIA amide terminus and the position 59 side chain, thereby significantly decreasing RegIIA affinity compared with wild-type $\alpha 3\beta 4$. In addition, non-conserved residues near the RegIIA binding site cannot compensate the affinity decrease of RegIIA with the $\beta 4$ Lys⁵⁹ to Thr mutation, which explains the observation that $\alpha 3\beta 4$ [K59T] is even significantly less sensitive to RegIIA than $\alpha 3\beta 2$.

Our hypothesis that the RegIIA Asn⁹/ $\beta 4$ Lys⁵⁹ contact is critical for the activity of RegIIA at $\alpha 3\beta 4$ is also corroborated by RegIIA alanine-scanning mutagenesis data. It has been shown that the [N9A]RegIIA analogue was inactive at $\alpha 3\beta 4$ and $\alpha 7$, but maintained inhibitory activity at $\alpha 3\beta 2$ (16). This study also noted that alanine analogues [N11A]RegIIA, [N12A]RegIIA, and [N11A,N12A]RegIIA had improved selectivity for $\alpha 3\beta 4$ compared with the native RegIIA. However, these analogues were less active than RegIIA at $\alpha 3\beta 4$ and the overall higher selectivity for $\alpha 3\beta 4$ resulted from considerable loss of activity at the $\alpha 3\beta 2$ and $\alpha 7$ nAChR subtypes. MD simulation also showed that for $\alpha 3\beta 2$, this was mainly due to destabilization of peptide contacts with multiple residues at the plus (+) and minus (-) interfaces (16).

α -Conotoxin LvIA remarkably revealed a considerable specificity for $\alpha 3\beta 2$ over $\alpha 6$ / $\alpha 3\beta 2\beta 3$ rat and human nAChRs, a rare feature not shared by other α -conotoxins such as RegIIA (32). As loops 1 of the two peptides are identical, these specificity differences must be conferred by the second loop in which three residues (11, 12, and 14) are different. Indeed, in our modeling most of the residues in the first loop of RegIIA form contacts with the principal $\beta 3$ subunit and most of the residues of the second loop form contacts with the complementary subunits (Table 4). At $\alpha 3\beta 2$ and $\alpha 3\beta 4$ nAChRs, we identified RegIIA residues Asn⁹, Asn¹¹, and Asn¹² as main determinants for interaction with the β subunit. We speculate that the residues at positions 11 and 12 primarily account for the differences in $\alpha 3\beta 2^*$ subtype sensitivity between LvIA and RegIIA.

Zhangsun *et al.* (31) also identified residues 111 and 119 in loop E of $\beta 2$ and $\beta 4$ subunits as determinants for LvIA potency, whereas for RegIIA, only residue 113 was responsible for the potency of the peptide in loop E. As mentioned previously, our modeling suggests that the β subunit specificity of RegIIA is conferred via interactions of its residues Asn¹¹ and Asn¹² with loop E of the receptor, specifically $\beta 4$ residue Arg¹¹³. As LvIA has different residues at these positions, the data on LvIA regarding key interacting residues with loop E (31) cannot be directly compared with RegIIA. However, as Asn⁹ is present in both RegIIA and LvIA, the interaction with $\beta 4$ Lys⁵⁹ likely occurs via the same mechanism leading to the similar observations in mutational analyses.

α -Conotoxin AuIB has been characterized as a selective inhibitor of the rat $\alpha 3\beta 4$ nAChR subtype (13), however, to our knowledge, its specificity profile at human nAChRs had not been evaluated. We tested AuIB at $\alpha 3\beta 2$ and $\alpha 3\beta 4$ nAChRs and selected mutants. AuIB appeared to be considerably less active at $\alpha 3\beta 4$, as 1 μ M AuIB did not inhibit ACh-evoked currents at this nAChR subtype and higher concentrations caused minimal inhibition, suggesting that the IC₅₀ is consid-

erably $>30 \mu\text{M}$ (Fig. 7, A and B). This is in contrast to the homologous rat $\alpha 3\beta 4$ nAChR where an IC_{50} of $0.75 \mu\text{M}$ was reported (13). Interestingly, we observed some inhibitory activity of AuIB at the $\text{h}\alpha 3\beta 2$ subtype. However, inhibition was slight with ACh-evoked current amplitude inhibited by $<50\%$ in the presence of $30 \mu\text{M}$ AuIB, similar to that observed for $\text{h}\alpha 3\beta 4$ (Fig. 7B).

Molecular modeling and docking simulation was also performed with α -conotoxin AuIB, indicating that many of the non-conserved residues in the complementary face of the agonist binding site are also involved in the binding of this peptide, although in a different way (Fig. 7, C and D, and Table 5). For example, residue Asp¹⁴ of AuIB forms a hydrogen bond with $\beta 4$ Arg¹¹³, whereas at the $\alpha 3\beta 2$ nAChR, $\beta 2$ Lys⁷⁹ interacts with Asp¹⁴. Residue Phe⁹ of AuIB interacts with several of the non-conserved residues, similar to Asn⁹ of RegIIA.

AuIB F9 has previously been identified as the key residue for the specific binding of AuIB to the rat $\alpha 3\beta 4$ nAChR (33). AuIB Phe⁹ interacts with Trp⁵⁷ and Lys⁵⁹ residues of the WLK pocket in rat $\beta 4$ loop D, (designated Trp⁵⁹ and Lys⁶¹ in this paper), most likely via π - π stacking due to the deep insertion of its aromatic ring (33). An alanine substitution substantially reduced $\alpha 3\beta 4$ inhibition and decreased subtype specificity. Additional AuIB analogues with other side chains at this position demonstrated that size, aromaticity, and hydrophobicity at position 9 of AuIB are important for interaction between the peptide and $\beta 4$ subunit of the rat $\alpha 3\beta 4$ pentamer (33).

Direct comparison of the orientation of RegIIA and AuIB at the $\alpha 3(+)\beta 4(-)$ binding site by MD simulation revealed that the $\beta 4$ subunit interface is largely unchanged in conformation, whereas the orientation of the two bound α -conotoxins differs significantly. RegIIA Asn⁹ is in close proximity to the $\beta 4$ Trp⁵⁷ and $\beta 4$ Lys⁵⁹ side chains, but the AuIB backbone is shifted relative to RegIIA, locating AuIB Phe⁹ outside of the WLK pocket (Fig. 7D). Thereby, AuIB Phe⁹ forms contact with $\beta 4$ Lys⁵⁹ and $\beta 4$ Leu¹¹⁹, but the crucial π - π interaction with $\beta 4$ Trp⁵⁷ is absent. As the tryptophan residue was indispensable for the inhibitory effect of AuIB at the rat homologue, and $\beta 4$ Lys⁵⁹ appeared to play an auxiliary role (33), the lack of interaction between AuIB F9 and $\beta 4$ Trp⁵⁷ could explain the loss of AuIB activity at the $\text{h}\alpha 3\beta 4$ subtype. At $\text{h}\alpha 3\beta 2$, our modeling revealed contacts between AuIB and $\beta 2$ Trp⁵⁷, Thr⁵⁹, and other residues (Fig. 7C and Table 5). Although the crucial interaction of AuIB Phe⁹ with the Trp⁵⁷ residue in loop D is present, residue Thr⁵⁹ does not form an effective binding pocket for AuIB Phe⁹, unlike $\beta 4$ Lys⁵⁹. The hydrophilic side chain of $\beta 2$ Thr⁵⁹ is likely unfavorable to interact with the aromatic phenyl ring of AuIB Phe⁹. In summary, our modeling offers an explanation for the lower inhibitory activity of AuIB at $\text{h}\alpha 3\beta 2$ and $\text{h}\alpha 3\beta 4$ nAChRs. The characteristic binding pocket for AuIB F9 formed by the two rat $\beta 4$ subunit loop D residues is absent in the human $\beta 2$ and $\beta 4$ counterparts, resulting in ineffective stabilization of AuIB.

In addition, nAChR subtype selectivity and wash-off kinetics has also been investigated for α -conotoxin BuIA (34, 35). A slow recovery from the BuIA block at $\alpha 3\beta 4$ compared with significantly faster recovery at $\alpha 3\beta 2$ has been observed in different species, including human (35). Mutational studies at rat $\beta 2$ and $\beta 4$ subunits have identified residues 59, 111, and 119 as

critical for the off-rate differences. Similar to our findings with RegIIA, the off-rate of BuIA was slower in the $\beta 2$ [T59K] mutant compared with wild-type $\alpha 3\beta 2$ (34).

Mutation of $\beta 2$ T59K has also been shown to be critical for dihydro- β -erythroidine and neuronal bungarotoxin sensitivity of $\alpha 3\beta 2$ (36), as well as affecting the affinity of the agonists, ACh and nicotine to the $\alpha 2\beta 2$ nAChR (37). The high selectivity of α -conotoxin MII for the $\alpha 3\beta 2$ nAChR subtype (>200 -fold less active at other nAChR subunit combinations) has been mapped to three sequence segments using $\alpha 3\beta 2$ chimeras. Within the segments, $\beta 2$ residue Thr⁵⁹ was identified as one factor determining the higher sensitivity of $\beta 2$ to MII compared with $\beta 4$ (38).

In summary, these findings confirm that subtype-selective nAChR antagonists often work through common mechanisms by interacting with the same structural components and sites on the receptor. Knowing the key residue interactions by which antagonists (such as α -conotoxins) can discern between structurally very similar nAChR subtypes provides us with the tools to design novel potent and highly subtype-selective drugs.

Here, we show that residues in loop 2 of α -conotoxins often form direct interactions with specific non-conserved residues in the agonist binding loops at the complementary interface of $\alpha 3\beta 2$ and $\alpha 3\beta 4$ nAChRs. In the future, generating toxin analogues with mutations in loop 2 to either improve or decrease binding to receptor key residues, such as Lys⁵⁹ or Arg¹¹³ of the $\beta 4$ subunit, might be a way to direct the selectivity of α -conotoxins to different subtypes of nAChRs. Given that $\text{h}\alpha 3\beta 4$ subtype-selective α -conotoxins are scarce, the design of new peptides targeting this nAChR subtype is desirable.

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Key Structural Determinants in the Agonist Binding Loops of Human $\beta 2$ and $\beta 4$ Nicotinic Acetylcholine Receptor Subunits Contribute to $\alpha 3\beta 4$ Subtype Selectivity of α -Conotoxins

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