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α -Conotoxin [S9A]TxID Potently discriminates between $\alpha 3\beta 4$ and $\alpha 6/\alpha 3\beta 4$ Nicotinic Acetylcholine Receptors

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Abstract

$\alpha 3\beta 4$ nAChRs have been implicated in various pathophysiological conditions. However, the expression profile of $\alpha 3\beta 4$ nAChRs and $\alpha 6/\alpha 3\beta 4$ nAChRs overlap in a variety of tissues. To distinguish between these two subtypes, we redesigned peptide **1** (α -conotoxin TxID), which inhibits $\alpha 3\beta 4$ and $\alpha 6/\alpha 3\beta 4$ nAChR subtypes. We systematically mutated **1** to evaluate analog selectivity for $\alpha 3\beta 4$ vs. $\alpha 6/\alpha 3\beta 4$ nAChRs expressed in *Xenopus laevis* oocytes. One analog, peptide **7** ([S9A]TxID), had 46-fold greater potency for $\alpha 3\beta 4$ versus $\alpha 6/\alpha 3\beta 4$ nAChRs. Peptide **7** had IC_{50} 's >10 μ M for other nAChR subtypes. Molecular dynamics simulations suggested that Ser-9 of TxID was involved in a weak hydrogen bond with $\beta 4$ Lys-81 in the $\alpha 6\beta 4$ binding site but not in the $\alpha 3\beta 4$ binding site. When Ser-9 was substituted by an Ala, this hydrogen bond interaction was disrupted. These results provide further molecular insights into the selectivity of **7** and provide a guide for designing ligands that block $\alpha 3\beta 4$ nAChRs.

TOC image

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*Supporting Information

Additional figures illustrating chromatographic and mass properties of analogs of α -conotoxin **1**. The two coordinate files correspond to the final frames of 50 ns molecular dynamics simulations of the complexes between conotoxin **1** and either $\alpha 3\beta 4$ or $\alpha 6\beta 4$ nAChR subtypes. The A and B chains are the ligand binding domain regions of the α and β subunits respectively. The C chain is the conotoxin **1**.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

INTRODUCTION

Nicotinic acetylcholine receptors (nAChRs) are ionotropic channels belonging to the Cys-loop superfamily of ligand-gated ion channels and have a pentameric protein structure.^{1,2} Generally, nAChRs are classified into muscle-type or neuronal-type receptors based on anatomical locations, though nAChR subunit expression is being increasingly reported in non-muscle and non-neural tissues. Muscle-type receptors consist of four different subunits assembled with an $(\alpha 1)_2\beta 1\delta \epsilon$ stoichiometry.³ By contrast, neuronal nAChRs are composed of a combination of α ($\alpha 2$ – $\alpha 10$) and β ($\beta 2$ – $\beta 4$) subunits, which can assemble to form homo- or hetero-pentamers. nAChRs are implicated in normal physiology and the pathophysiology of a number of disease states, including pain, Parkinson's disease, Alzheimer's disease, schizophrenia, epilepsy, dementia and cancer.^{3,4} $\alpha 4\beta 2^*$ (* indicates possible presence of additional subunits) nAChRs are the most abundant heteromeric subtype in the mammalian brain and receptors containing the $\alpha 4$ nAChR subunit are widely distributed.⁵ In contrast receptors containing the $\alpha 6$ nAChR subunit have a more restricted distribution with concentration in catecholaminergic and visual pathways.⁶ $\alpha 4\beta 2^*$ and $\alpha 6\beta 2^*$ are both strongly implicated in tobacco addiction and compounds with activity at these receptor subtypes are utilized or in development as anti-smoking drugs.⁴ nAChR subtypes that contain the $\alpha 3$ subunit are present in autonomic ganglia and modulate cardiovascular functions. The $\alpha 3^*$ subtypes of nAChRs are also implicated in modulating pain sensations.⁷ In addition, the $\alpha 3\beta 4$ nAChR has been shown to be associated with nicotine addiction and drug abuse.^{8,9} Furthermore, several studies have shown that hypothalamic $\alpha 3\beta 4$ nAChRs are implicated in nicotinic-induced decreases in food intake, and might therefore provide clues to treating food overconsumption and obesity.¹⁰ Thus, strategies to selectively modulate $\alpha 3\beta 4$ nAChR function are of considerable interest since all the available ligands lack good selectivity between $\alpha 3\beta 4$ and the closely related $\alpha 6\beta 4$ nAChR subtypes, limiting our understanding of the physiological roles of these receptors.

Cone snails are venomous marine molluscs that have evolved a diverse array of structured peptide toxins for capturing their prey or defending against predators. Because conotoxins are potent antagonists of a range of voltage- or ligand-gated ion channels, they are widely used as valuable tools in ion channel research, and several have direct diagnostic or therapeutic potential. Their diverse sequences are documented in the ConoServer database.¹¹ Most of the pharmacologically characterized conotoxins are nAChR antagonists, and are therefore classified as α -conotoxins. The α -conotoxins typically comprise 12–20 amino acids and have exquisite nAChR subtype selectivity, thus enabling dissection of the functional roles of nAChR subtypes. Peptide **1** (α -Conotoxin TxID) cloned from *Conus textile* is one of the most potent $\alpha 3\beta 4$ nAChR antagonists, and is potentially a valuable tool for elucidating the diverse roles of $\alpha 3\beta 4$ nAChRs.¹² In this study we used positional-scanning mutagenesis of peptide **1** (Figure 1) to identify critical residues that confer potency for $\alpha 3\beta 4$ nAChRs. We counter-screened analogs against $\alpha 6/\alpha 3\beta 4$ nAChRs ($\alpha 6/\alpha 3$ designates a chimeric subunit that contains the extracellular ligand binding domain of $\alpha 6$ linked to the membrane embedded and intracellular portions of the $\alpha 3$ subunit).¹³ An analog with significantly improved selectivity for $\alpha 3\beta 4$ versus $\alpha 6/\alpha 3\beta 4$ nAChRs was identified.

RESULTS

Synthesis of wild type peptide 1 and analogs

Peptide **1** is a 15-amino acid peptide, which belongs to the α -4/6 subclass of conotoxins and its nucleic acid encoding sequence was first determined from *Conus textile*.¹² In addition to its presumed natural function in prey capture, it is a potent and selective antagonist of rat α 3 β 4 nAChRs. Peptide **1** also shows activity at rat α 6/ α 3 β 4 nAChRs, albeit with 10-fold less potency than at the α 3 β 4 subtype. To understand the structure–activity relationships of peptide **1**, we designed and synthesized a suite of Ala-substituted peptide **1** analogs (**2**–**16**). In the course of these experiments we noticed that the methionine residue of peptide **1** at position 11 (Met-11) was easily oxidized into a methionine sulfoxide residue, leading to a reduction of activity (data not shown). We therefore substituted Met-11 with isoleucine. The side chains of Met and Ile amino acids are both hydrophobic but Ile is not susceptible to oxidation. Sequence comparison (Figure 1) between peptide **1** and **17**, which is similar in structure but less potent at the α 3 β 4 subtype, suggested that position 14 could be important for affinity.¹⁴ We thus tested the importance of position 14 by substituting Ile-14 with an Asp (as found in peptide **17**), which is negatively charged, as well as an Arg residue, which is positively charged, and a Tyr residue, which is a bulky aromatic residue with hydrogen bond donor-acceptor properties often found on the surface of proteins.

For all of these mutants the peptide chain was successfully synthesized using Fmoc chemistry. The Cys residues were orthogonally protected using acid labile S-trityl (Trt) and acid-stable S-acetamidomethyl (Acm) groups. After cleavage of the assembled peptide chain from the resin, the Trt protecting groups on Cys I and Cys III were removed and the cysteines oxidized by treatment with ferricyanide. The second disulfide bridge was then formed between Cys-II and Cys-IV after Acm deprotection using iodine oxidation: the monocyclic conopeptide was trickled into an equal volume of a solution of iodine (10 mM) in H₂O/trifluoroacetic acid/acetonitrile (78:2:20 by volume). The reaction was allowed to proceed for 10 min and then terminated by the addition of ascorbic acid. The solution was diluted 20-fold with 0.1% trifluoroacetic acid and the bicyclic peptide was purified by high-performance liquid chromatography (HPLC).¹⁵ This two-step oxidative folding strategy resulted in a single disulfide bond isomer (Figure 2, Figure S1). The observed molecular mass (1488.6 Da) of peptide **1** was consistent with the calculated mass (1488.6 Da) (Figure 2B). Similarly, the observed molecular mass of peptide **7** was 1472.6 Da, consistent with the calculated mass (1472.6 Da) (Figure 2D). Other mutants were synthesized by the same method and their HPLC and mass spectrometry (MS) profiles are provided in Supplemental Information Figure S1.

Electrophysiological Assay of Mutagenesis of Rat nAChRs Expressed in *Xenopus laevis* Oocytes

To better understand which positions are responsible for the activity of peptide **1**, we performed alanine scanning mutagenesis. Except for the four Cys residues and Ala-10, each of the original residues was substituted by Ala. The peptide **1** analogs were tested on nAChR subtypes at concentrations up to 10 μ M. The activities of peptide **4** ([H5A]TxID), **5** ([P6A]TxID), **6** ([V7A]TxID), **8** ([M11A]TxID) and **10** ([P13A]TxID) analogs at the α 3 β 4

nAChR subtype were reduced by greater than 1000-fold compared to native peptide **1**. The activities of these five analogs on $\alpha 6/\alpha 3\beta 4$ nAChRs were also abolished. In contrast, several analogs retained high potency for $\alpha 3\beta 4$ nAChRs and $\alpha 6/\alpha 3\beta 4$ nAChRs. We therefore investigated the concentration-response relationships of peptide **1** and those analogs that showed antagonist activity at the $\alpha 3\beta 4$ and $\alpha 6/\alpha 3\beta 4$ nAChR subtypes (Table 1). Replacing the first glycine residue with an alanine substantially decreased potency at the $\alpha 3\beta 4$ subtype. The G1A substitution also caused a decreased inhibition at the $\alpha 6/\alpha 3\beta 4$ nAChR by 8-fold compared to the parent peptide. The activities of **3**, **9** and position 14 analogs displayed similar potency at the $\alpha 3\beta 4$ subtype compared to the parent peptide. Peptide **12**, in which the easily oxidized methionine residue was substituted by an isoleucine amino acid, had 20-fold reduced potency at $\alpha 3\beta 4$ nAChR, with an IC_{50} of 74.9 nM. By contrast, peptide **3**, **9**, **12** and the four position 14 analogs globally retained activity at the $\alpha 6/\alpha 3\beta 4$ nAChR subtype. None of the substitutions at positions 1, 4, 11, 12, 13 and 14 resulted in increased selectivity for the $\alpha 3\beta 4$ subtype over the $\alpha 6/\alpha 3\beta 4$ subtype compared to peptide **1**.

Remarkably, the S9A substitution had little impact on the activity at $\alpha 3\beta 4$ but led to a 5-fold decrease in inhibition of $\alpha 6/\alpha 3\beta 4$, creating a 46-fold selective compound for $\alpha 3\beta 4$ compared to $\alpha 6/\alpha 3\beta 4$ nAChRs. Peptide **7** is the only compound in this study showing a higher $\alpha 3\beta 4$ -selectivity than the parent peptide **1** (Figures 3 and 4). Representative traces of ACh-evoked currents for $\alpha 3\beta 4$ and $\alpha 6/\alpha 3\beta 4$ nAChRs inhibited by peptide **1** and **7** are shown in Figure 4.

As described above, substituting Ser-9 by an Ala amino acid in peptide **1** increased selective inhibitory activity for the $\alpha 3\beta 4$ subtype over $\alpha 6/\alpha 3\beta 4$ subtype. We subsequently demonstrated that peptide **7** did not inhibit a range of other subtypes at 10 μ M, including $\alpha 7$, $\alpha 9\alpha 10$, $\alpha 1\beta 1\delta\epsilon$, $\alpha 2\beta 2$, $\alpha 2\beta 4$, $\alpha 3\beta 2$, $\alpha 4\beta 2$, and $\alpha 4\beta 4$ (Table 2).

NMR Spectroscopy

NMR spectroscopy analysis revealed that peptide **7** exists as two isomers in aqueous solution at 308 K in a ratio of approximately 70:30. The conformational heterogeneity appears to arise from cis-trans isomerization of the peptide bonds preceding the Pro residues at positions 6 and 13. Isomer 1 was assigned as the trans-trans isomer based upon NOEs observed between the $H\alpha_{i-1} - H\delta_{Pi}$ protons of Pro6 and Pro13 and their preceding residues. Complete assignment of the spectral peaks corresponding to this isomer was possible, with the exception of the N-terminal glycine residue. By contrast, isomer 2 could only be partially assigned, particularly towards the N- and C-termini of the peptide. This isomer was determined to be a cis-trans isomer because of the lack of any NOE between $H\alpha_5$ and $H\delta_6$ protons, and consistency with previous findings for the wild-type peptide.¹²

Secondary shift analysis (Figure 5), was used to compare the secondary structure elements of peptide **7** with those of peptide **1**.¹² Deviations of α H shifts greater than 0.1 ppm from random coil values¹⁶ are typically indicative of structured peptides, with positive values present for β -type structure and negative values for helical structures. Figure 5 reveals no significant difference in the secondary structure of the trans-trans form (isomer 1) of **7** compared to the trans-trans isomer of **1**. Indeed, the mutation of Ser-9 to Ala-9 appears to

strengthen the helical nature across the middle of this peptide. By contrast, the *cis-trans* isomer of both peptide **1** and its [S9A] derivative, both appear to adopt more a random coil structure (data not shown).

DISCUSSION

In this paper we report the synthesis, electrophysiological characterization and structural characterization of a series of mutants of the α -conotoxin **1**. First, using alanine scanning and site-directed mutagenesis of the non-cysteine residues, we identified five key residues in the peptide **1** sequence, i.e. His-5, Pro-6, Val-7, Met-11 and Pro-13, which when substituted to Ala caused total loss of inhibition at both $\alpha 3\beta 4$ and $\alpha 6/\alpha 3\beta 4$ nAChRs. Compared to native peptide **1**, the substitution G1A caused a 17-fold decrease in activity at the $\alpha 3\beta 4$ nAChR. Other Ala substitutions, i.e. of residues Ser-4, Ser-9, Ser-12 or Ile-14, resulted in more modest reductions in activity for the $\alpha 3\beta 4$ nAChR (Table 1). Notably, the S9A substitution resulted in little or no loss of potency for the $\alpha 3\beta 4$ nAChR. When these mutants were tested on rat $\alpha 6/\alpha 3\beta 4$ nAChRs, there was a general decrease in activity. Ser for Ala substitution in the 9th position resulted in a ~46-fold preference for $\alpha 3\beta 4$ versus the very closely related $\alpha 6/\alpha 3\beta 4$ nAChR subtype. Recently, the $\alpha 3\beta 4$ subtype has received considerable attention by being implicated in lung cancer and in nicotine addiction.⁴ Several α -conotoxins have been identified as antagonists of the $\alpha 3\beta 4$ nAChR. However, most of them target a broad range of subtypes, especially for $\alpha 6$ containing subtypes, as shown in Table 3. α -Conotoxin **17** from *Conus aulicus* was the first conopeptide that selectively blocks $\alpha 3\beta 4$ nAChR, but its low potency has limited its use in physiological studies.¹⁴ [N11,12A]RegIIA, a mutant of α -conotoxin RegIIA blocks $\alpha 3\beta 4$ nAChR with an IC₅₀ of 370 nM, and also inhibits the $\alpha 6/\alpha 3\beta 4\beta 3$ nAChR with an IC₅₀ of 5100 nM.¹⁷ Interestingly, a α -4/4-conotoxin BuIA analog, [T5A, P6O]BuIA, is a potent antagonist of $\alpha 6/\alpha 3\beta 4$ nAChRs with IC₅₀ 58.1 nM whereas it blocks rat $\alpha 3\beta 4$ nAChRs with an IC₅₀ of 1200 nM (Table 3).¹⁸ The current study suggests that **7** has an opposite nAChR subunit selectivity to that of [T5A, P6O]BuIA, which may reveal further insights into how conotoxins discriminate between $\alpha 3\beta 4$ and $\alpha 6/\alpha 3\beta 4$ nAChRs. Another BuIA analogue, TP-2212-59, in which two amino acids are substituted by 2-aminobutyric acid and norvaline, is the most potent $\alpha 3\beta 4$ nAChR antagonist with an IC₅₀ of 2.3 nM,¹⁹ which is similar to native peptide **1** and **7**. But the activity of TP-2212-59 was not reported for $\alpha 6$ -containing nAChRs. Thus, Peptide **7** has the greatest selectivity for the $\alpha 3\beta 4$ vs. $\alpha 6/\alpha 3\beta 4$ nAChR compared to previously characterized α -conotoxins. We are not aware of any compounds that have greater selectivity than peptide **7** for $\alpha 3\beta 4$ vs. $\alpha 6/\alpha 3\beta 4$ nAChRs.

NMR structural studies of peptide **7** revealed the presence of two major conformational isomers, as was found for peptide **1**.¹² The isomerization derives from *cis-trans* conformational interchange of the His-5/Pro-6 peptide bond, with Ser-12/Pro-13 apparently fixed in the *trans* form. Isomer 1 corresponds to a *trans-trans* arrangement of the two Pro residues whereas isomer 2 has a *cis-trans* configuration. A similar pattern of isomerisation was observed in the wild type peptide, although interestingly, the mutation of Ser-9 to Ala-9 appears to slightly favor an increased population of the *trans-trans* isomer versus the *cis-trans* isomer (70:30 in the mutant vs 40:60 in the wild type peptide, respectively). Secondary

shift analysis confirms that the major (i.e. most populated) isomer of peptide **7** adopts a solution structure that is highly similar to isomer 1 of peptide **1**. That structure comprises a short 3₁₀-helix that spans residues 8–10, preceded by a short turn, and is braced by the globular disulfide connectivity. The turn in loop 1 followed by a helical segment is a conserved motif displayed by all α -conotoxins, and the interaction of this motif with nAChRs is one of the best understood interactions between toxins and proteins,²³ and for example, this motif is also displayed by snake toxins binding to nAChRs. The binding mode of this motif is similar in crystal structures of α -conotoxins (PDB identifiers 2br8, 2byp, 2c9t, 2uz6, 4ez1) and snake toxins (PDB identifiers 1yi5, 2qc1, 4uy2, 4hqp) binding to the acetylcholine binding protein, which is a structural surrogate of the ligand binding domain of nAChRs. The turn is tightly packed at the bottom of the acetylcholine binding pocket in the “aromatic box”, a conserved feature of the agonist binding site. Thus isomer 1 is the likely binding conformation of peptide **1**. In contrast to isomer 1, isomer 2 of peptide **1** does not display this structural motif,¹² suggesting that it does not bind the orthosteric binding site. We therefore created molecular models of the complex between peptide **1** isomer 1 and nAChRs to study the functional impact of substitutions.

A comparison of molecular models of **1**/ α 3 β 4 and **1**/ α 6 β 4 complexes suggests that the binding modes are slightly different despite nearly all positions in the binding site being conserved between the two receptors (Figure 6A–B). This difference in binding modes originates from a slightly different shape of the binding sites which, in turn, probably arises from sequence differences between the α 3 and α 6 subunits at positions proximal to the binding site. During the molecular dynamics simulation, the side chain of peptide **1** Ser-9 was involved in a weak hydrogen bond with β 4 Lys-81 in the α 6 β 4 binding site but not in the α 3 β 4 binding site (Figure 6C). The substitution of Ser by an Ala at position 9 of peptide **1** would disrupt this hydrogen bond interaction, and the experimentally measured five-fold decreased potency, accounting for a loss of around ~1 kcal/mol at 300 K, is consistent with a loss of a hydrogen bond.²⁴ By contrast, the hydroxyl group of peptide **1** Ser-4 in the α 3 β 4 binding site is not involved in any interaction with the receptor, and therefore the S9A substitution is predicted to have no impact, which is also consistent with experimental measurements.

Substitution of Gly-1 by an Ala led to a 17-fold or 8-fold reduction of inhibition of α 3 β 4 or α 6/ α 3 β 4, respectively. The models suggest a possible charge interaction at the interface with the negatively charged Asp-192 located on the β 4 subunit, and this interaction could be weakened by a change of backbone conformation of **1** compared to the parent peptide. The substitution of Met-11 by Ile resulted in 20-fold decreased activity at the α 3 β 4 nAChR but did not reduce inhibition of the α 6/ α 3 β 4 nAChR. According to the model, Met-11 contacts Cys-218 of the C-loop of the α 3 subunit and the substitution M11A would probably result in a change of binding mode because of the steric hindrance caused by the larger side chain. By contrast, Met-11 in the α 6 β 4 model could be substituted by an Ile residue without creating any steric clash with the α 6 subunit in its bound conformation with wild-type peptide **1**, suggesting that this substitution would not have a detrimental effect.

In conclusion, our study has identified critical residues for specific interactions of peptide **1** with the α 3 β 4 nAChR. We discovered and characterized a new mutant peptide **7** that is 46-

fold more potent at blocking $\alpha 3\beta 4$ than $\alpha 6/\alpha 3\beta 4$ nAChR subtypes. This selectivity provides a template for the further design and development of ligands to discriminate between $\alpha 3\beta 4$ and $\alpha 6/\alpha 3\beta 4$ nAChR subtypes.

EXPERIMENTAL SECTION

Materials

Protected Fmoc-amino acid derivatives were purchased from GL Biochem (Shanghai, China). The following side chain protected amino acids were used: Cys(Acm), Cys(Trt), His(Trt), Tyr(tBu), Trp(Boc), Arg(Pbf), Asp(OtBu), Ser(tBu). All other Fmoc amino acids were unprotected. Dimethylformamide (DMF), dichloromethane (DCM), diisopropylethylamine (DIEA), 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), ethanedithiol(EDT), thioanisole for peptide synthesis were from Applied Biosystems (Foster City, CA, USA) and GL Biochem (Shanghai, China). Acetonitrile (HPLC grade) was purchased from Fisher Scientific (Pittsburg, PA, USA). Trifluoroacetic acid was from Tedia Company (Fairfield, OH, USA). All other chemicals used were of analytical grade. Acetylcholine chloride, atropine, and bovine serum albumin were obtained from Sigma (St. Louis, MO, USA). Reversed-phase HPLC analytical Vydac C18 (5 μ m, 4.6 mm $\times$ 250 mm) and preparative C18 Vydac columns (10 μ m, 22 mm $\times$ 250 mm) were obtained from Grace Vydac (Hesperia, CA, USA). Clones of rat nAChR subunits and mouse muscle subunits ($\alpha 1\beta 1\delta\epsilon$) were kindly provided by S. Heinemann (Salk Institute, San Diego, CA, USA).

Chemical Synthesis of peptide 1 and its mutants

All of the α -conotoxin **1** analogs were synthesized on an automatic ABI 433A peptide synthesizer using the ABI FastMoc protocols Fmoc (N-(9-fluorenyl)methoxycarbonyl) amino acid derivatives, activated in situ using 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate/1-hydroxybenzotriazole and N,N9-diisopropylethylamine in N-methylpyrrolidinone, were used in the coupling steps. The peptide was deprotected and cleaved from the resin by treatment with reagent K (trifluoroacetic acid/water/ethanedithiol/phenol/thioanisole; 90:5:2.5:7.5:5, v/v/v/v/v) for 2 h at room temperature. The released peptides were precipitated and washed several times with cold ether. Then crude peptides dissolved in B60 (60% CH₃CN, 40% H₂O, 0.05% TFA). Then these peptides were purified by reversed phase-high performance liquid chromatography (RP-HPLC) on a Vydac C18 column using a gradient of 10–50% buffer B for 40 min and the eluant was monitored at 214 nm. Electrospray-mass spectroscopy (ESI-MS) confirmed the molecular mass of the fractions collected. A two-step oxidation protocol was used to synthesize α -conotoxin **1** and mutants as described previously.¹⁵ In this protocol, linear and folded peptides were purified by HPLC on a reversed-phase C18 Vydac column. HPLC elution conditions included a linear gradient of 10–40% Buffer B over 20 min. Buffer A was 0.1% trifluoroacetic acid in H₂O. Buffer B was 0.05% TFA, 90% acetonitrile in H₂O. Absorbance was monitored at 214 nm. The purity of the monocyclic and bicyclic peptides was determined by monitoring absorbance at 214 nm during HPLC ($\geq 95\%$ purity). Mass spectrometry was utilized to confirm the identity of the products.

Electrophysiology

The cRNAs of rat nAChR subunits were obtained by in vitro transcription using the mMessage mMachine SP6 kit (Ambion, Austin, TX, USA). The cRNAs were purified using the MEGAClear™ kit (Ambion). The concentration of each cRNA was determined by Smart Spec™ plus Spectrophotometer (Bio-Rad). Oocytes were harvested and injected with cRNA encoding nAChR subunits as described previously.²⁵ Briefly, oocytes were transferred to the recording chamber (~30 μ L in volume) and gravity-perfused at 2 mL/min with ND-96 buffer (96 mM NaCl, 2.0 mM KCl, 1.0 mM MgCl₂, 1.8 mM CaCl₂, 5 mM HEPES, pH 7.1–7.5) containing 1 μ M atropine and 0.1 mg/mL bovine serum albumin (BSA). ACh-gated currents were obtained with a two-electrode voltage-clamp amplifier (model OC-725B, Warner Instruments, Hamden, CT, USA). The oocytes were clamped at a holding potential of –70 mV. Oocytes were gravity-perfused with standard ND-96 solution and stimulated with 1-s pulses of ACh once every minute. For screening the specific of nAChR subtype, the toxins concentration was 10 μ M or lower. Once a stable baseline was achieved, added 3 μ L of different concentration toxin (0.1 nM–10 μ M) to the chamber and waited for 5 min, then applied perfusion system, during which 1-s pulses of 10 μ M ACh were applied every one minute. The concentration-response data were fitted to the equation, % response = $100 / \{1 + ([\text{toxin}] / \text{IC}_{50})^{\text{nH}}\}$, where nH is the Hill coefficient, by nonlinear regression analysis using GraphPad Prism (GraphPad Software, San Diego, CA, USA). Each data point of a concentration-response curve represents the average $\pm$ S.E.M. of 6 to 21 oocytes.

NMR Spectroscopy

NMR spectra were recorded as described previously for peptide **1**.¹² Peptide **7** (0.9 mg) was dissolved in 500 μ L of 10% D₂O/90% H₂O (~pH 4) and spectra were recorded at 308 K on a Bruker Advance 600 MHz spectrometer. One-dimensional proton spectra and two-dimensional TOCSY (mixing time of 80 ms) and NOESY (mixing time of 200 ms) spectra were referenced to internal 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) at 0 ppm.

Molecular modeling

Models of the interactions between peptide **1** and rat $\alpha 3\beta 4$ or $\alpha 6\beta 4$ nAChR ligand binding domains were generated using the same strategy as previously.²⁶ Initial models were built by homology using Modeller9v13²⁷ and two templates: the crystal structure of AChBP bound to conotoxin TxIA variant (PDB identifier 2uz6²⁸) and the crystal structure of the isolated human $\alpha 9$ subunit (PDB identifier 4d01²⁹). The two nAChR subtypes were assumed to have a stoichiometry of two α subunits and three β subunits and the toxin was assumed to occupy the orthosteric binding site, which is located at the interface between an α (principal) subunit and a β (complementary) subunit. These models were then refined using molecular dynamics (MD) with the amber ff99SB-ILDN³⁰ force field and the Gromacs 5.1 MD engine.³¹ The system was solvated in a dodecahedron box with ~28,000 TIP3P water molecules and sodium and chloride ions were added to reach a concentration of 150 mM NaCl. Additional sodium ions were added to neutralize the system. The system was equilibrated over 5 ns while progressively releasing position restraints on the protein atoms. The production run lasted 50 ns and employed a velocity rescaling scheme thermostat³² to maintain the temperature at 300 K and a Parrinello-Rahman barostat³³ to maintain

atmospheric pressure. The particle mesh Ewald algorithm³⁴ was used to simulate long-range electrostatic interactions. Three MD simulations were carried out for each of the two systems.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

CTx	conotoxin
nAChR	nicotinic acetylcholine receptor
TFA	trifluoroacetic acid
RP-HPLC	reversed-phase high-performance liquid chromatography
ESI-MS	Electrospray-mass spectroscopy
BSA	bovine serum albumin
DMF	Dimethylformamide
DCM	dichloromethane
DIEA	diisopropylethylamine
HBTU	2-(1H-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
EDT	ethanedithiol
Fmoc	N-(9-fluorenyl)methoxycarbonyl
AChBP	acetylcholine-binding protein

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Name	CN ^a	Sequence														
		I	II	III				IV								
TxID	1	G	C	C	S	H	P	V	C	S	A	M	S	P	I	C*
[G1A] TxID	2	A	C	C	S	H	P	V	C	S	A	M	S	P	I	C*
[S4A]TxID	3	G	C	C	A	H	P	V	C	S	A	M	S	P	I	C*
[H5A]TxID	4	G	C	C	S	A	P	V	C	S	A	M	S	P	I	C*
[P6A]TxID	5	G	C	C	S	H	A	V	C	S	A	M	S	P	I	C*
[V7A]TxID	6	G	C	C	S	H	P	A	C	S	A	M	S	P	I	C*
[S9A]TxID	7	G	C	C	S	H	P	V	C	A	A	M	S	P	I	C*
[M11A]TxID	8	G	C	C	S	H	P	V	C	S	A	A	S	P	I	C*
[S12A]TxID	9	G	C	C	S	H	P	V	C	S	A	M	A	P	I	C*
[P13A]TxID	10	G	C	C	S	H	P	V	C	S	A	M	S	A	I	C*
[I14A]TxID	11	G	C	C	S	H	P	V	C	S	A	M	S	P	A	C*
[M11I]TxID	12	G	C	C	S	H	P	V	C	S	A	I	S	P	I	C*
[S9A,M11L]TxID	13	G	C	C	S	H	P	V	C	A	A	L	S	P	I	C*
[I14R]TxID	14	G	C	C	S	H	P	V	C	S	A	M	S	P	R	C*
[I14Y]TxID	15	G	C	C	S	H	P	V	C	S	A	M	S	P	Y	C*
[I14D]TxID	16	G	C	C	S	H	P	V	C	S	A	M	S	P	D	C*
AuIB	17	G	C	C	S	Y	P	P	C	F	A	T	N	P	D	C*

Figure 1.

The sequences of peptide **1** and its analogs. The disulfide connectivity of Cys (I–III, II–IV) is schematically illustrated with lines connecting the Cys residues. Substituted amino acids that were mutated to explore structure-activity relationships are marked in red. (The * indicates a C-terminal amide). Also shown for comparison is peptide **17** (α -conotoxin AuIB),¹⁴ for which we compare structure and activity data. ^aCompound number.

Figure 2.

HPLC chromatograms and mass spectra of peptide **1** and **7**. The peptides were analyzed on a reversed phase analytical Vydac C18 HPLC column using a linear gradient of 10% buffer B to 40% buffer B over 20 min, where buffer A = 0.65% trifluoroacetic acid (TFA), remainder H₂O, B = 0.5% TFA, 90% acetonitrile, remainder H₂O. Absorbance was monitored at 214 nm. (A) HPLC chromatogram of peptide **1**; (B) electrospray ionization mass spectrometry (ESI-MS) data for peptide **1** with observed monoisotopic mass of 1488.6 Da; (C) HPLC chromatogram of peptide **7**; (D) ESI-MS data for peptide **1** with an observed monoisotopic mass of 1472.6 Da.

Figure 3.

Concentration-response analysis for inhibition of $\alpha 3\beta 4$ and $\alpha 6/\alpha 3\beta 4$ nAChRs by peptide **1** and **7**. (A) Peptide **1** selectively blocks $\alpha 3\beta 4$ nAChR vs $\alpha 6/\alpha 3\beta 4$ nAChR by only 10-fold; (B) Peptide **7** selectively blocks $\alpha 3\beta 4$ nAChR vs $\alpha 6/\alpha 3\beta 4$ nAChR by 46-fold. The IC_{50} values are in Table 1. Error bars represent S.E.M. values from 4–5 oocytes for each experimental determination.

Figure 4.

Block by peptide **1** and **7** of rat $\alpha 3\beta 4$ and $\alpha 6/\alpha 3\beta 4$ nAChRs. *Xenopus laevis* oocytes expressing the indicated nAChRs were voltage-clamped at -70 mV and subjected to a 1 s pulse of ACh every minute as described in the Experimental Section. A representative response in a single oocyte is shown. After control responses to ACh, the oocyte was exposed to 10 nM or 100 nM toxin for 5 min (arrow). The toxin was then washed out, and the response to ACh was again measured. “C” indicates control responses to ACh.

Figure 5.

Comparison of NMR spectroscopy of the peptide **7** and wild-type peptide **1**. (A) Secondary chemical shift analysis of peptide **7** isomer 1 (black) compared to that of isomer 1 of peptide **1** (grey). (B) Homology models of the two peptide **7** isomers (grey) compared to the NMR solution structures of the wild-type isomers (white).¹² The amino acid sequence of peptide **7** is displayed, with the substituted residue for the mutant shown in brackets. The homology models were refined using extensive energy minimization in explicit water. The models suggest that the three-dimensional structure of the isomers is essentially not affected by the S9A substitution.

Figure 6.

Comparison of molecular models of the binding mode of peptide **1** at the $\alpha 3\beta 4$ (A) and $\alpha 6\beta 4$ (B) interface, and distance between side chain hydroxyl of peptide **1** Ser-9 and side chain nitrogen of $\beta 4$ Lys-81 during 50 ns molecular dynamics (MD) simulations. The molecular models shown are the final frames of the 50 ns MD simulations, and the $\alpha 3$ subunit is drawn in pink, the $\alpha 6$ is in blue and the $\beta 4$ subunits are in green. A hydrogen bond between Ser-9 and Lys-81 is indicated with a dotted line. Positions of $\beta 4$ subunits are numbered according to the full-length sequence of rat $\beta 4$ subunit precursor sequence (UniProt identifier P12392).

Table 1

Potencies of peptide **1** and its analogs on rat $\alpha 3\beta 4$ and $\alpha 6/\alpha 3\beta 4$ nAChR subtypes expressed in *Xenopus laevis* oocytes

Peptide	IC ₅₀ (nM) (95% CI) $\alpha 3\beta 4$	n ^H	$\alpha 3\beta 4$ IC ₅₀ ratio relative to 1	IC ₅₀ (nM) (95% CI) $\alpha 6/\alpha 3\beta 4$	n ^H	$\alpha 6/\alpha 3\beta 4$ IC ₅₀ ratio relative to 1	Selectivity ^a
1	3.6 (1.8–7.3)	0.71 (0.35–1.10)	1.0	33.9 (23.6–48.7)	1.10 (0.78–1.50)	1.0	9.4
2	61.9 (32.4–118.0)	0.59 (0.36–0.83)	17	278 (154–503)	1.0 (0.50–1.50)	8.2	4.5
3	10.8 (8.6–13.4)	1.16 (0.763–1.56)	3.0	64.1 (41.6–98.7)	0.87 (0.54–1.21)	1.9	5.9
4	>10 μ M	–	–	>10 μ M	–	–	–
5	>10 μ M	–	–	>10 μ M	–	–	–
6	>10 μ M	–	–	>10 μ M	–	–	–
7	3.9 (2.5–5.9)	0.74 (0.52–0.96)	1.1	178.1 (137.0–231.5)	0.85 (0.67–1.03)	5.2	46
8	>10 μ M	–	–	>10 μ M	–	–	–
9	17.4 (8.6–3.5)	1.12 (0.26–1.97)	4.8	39.3 (25.6–59.8)	1.03 (0.66–1.41)	1.2	2.3
11	16.1 (9.1–28.5)	1.05 (0.39–1.70)	4.4	45.8 (33.7–62.2)	1.04 (0.77–1.32)	1.3	2.8
12	74.9 (55.0–102.1)	1.03 (0.75–1.31)	21	50.4 (31.3–81.2)	0.85 (0.52–1.18)	1.5	0.67
13	30.7 (14.1–66.8)	0.85 (0.34–1.37)	8.4	101.0 (55.8–183.2)	0.91 (0.45–1.37)	3.0	3.3
10	>10 μ M	–	–	>10 μ M	–	–	–
14	9.8 (6.6–14.4)	1.33 (0.387–2.26)	2.7	40.8 (32.9–50.5)	0.84 (0.69–0.98)	1.2	4.2
15	10.0 (6.4–15.6)	0.75 (0.52–0.98)	2.7	38.7 (23.0–65.0)	0.68 (0.42–0.93)	1.1	3.9
16	9.2 (5.0–16.9)	0.75 (0.38–1.12)	2.5	67.4 (42.8–106)	1.02 (0.53–1.50)	2.0	7.3

IC₅₀ values (nM) with 95% CI and Hill slope (n^H) obtained from concentration–response curves for peptide **1** and analogues at $\alpha 3\beta 4$ and $\alpha 6/\alpha 3\beta 4$ nAChR subtypes. All data represent mean of n = 6–21 oocytes.

^aRatio of $\alpha 6/\alpha 3\beta 4$: $\alpha 3\beta 4$ IC₅₀ values.

Table 2

IC₅₀ for block of nAChR subtypes by α -conotoxins **1** and peptide **7**.

Peptide	Subtype	IC ₅₀ [nM] (95% CI)
1	$\alpha 3\beta 4$	3.6 (1.8–7.3)
	$\alpha 6/\alpha 3\beta 4$	33.9 (23.6–48.7)
7	$\alpha 3\beta 4$	3.9 (2.5–5.9)
	$\alpha 6/\alpha 3\beta 4$	178.1 (137.0–231.5)
7	$\alpha 7$	>10,000 ^a
	$\alpha 9\alpha 10$	>10,000 ^a
	Ma1 β 1 δ e	>10,000 ^a
	$\alpha 2\beta 2$	>10,000 ^a
	$\alpha 2\beta 4$	>10,000 ^a
	$\alpha 3\beta 2$	>10,000 ^a
	$\alpha 4\beta 2$	>10,000 ^a
	$\alpha 4\beta 4$	>10,000 ^a

^aLess than 50% block at 10 μ M. All receptors are rat except for $\alpha 1\beta 1\delta$ e, which is mouse.

Table 3

α -Conotoxins that target the $\alpha 3\beta 4$ and $\alpha 6/\alpha 3\beta 4$ nAChRs.

Name	Sequence	Source	IC ₅₀ [nM] $\alpha 3\beta 4$	IC ₅₀ [nM] $\alpha 6/\alpha 3\beta 4$	Ratio ^a	nAChR subtype selectivity	Ref.
Peptide 1	GCCHHPVCSAMSP-IC [*]	<i>C. textile</i>	3.6	33.9	9.4	$\alpha 3\beta 4 > \alpha 6/\alpha 3\beta 4 > \alpha 2\beta 4$	12
Peptide 7	GCCHHPVCAAMSP-IC [*]	synthetic	3.9	178	46	$\alpha 3\beta 4 > \alpha 6/\alpha 3\beta 4$	This work
Peptide 17	GCCHYPFCFATNP-DC [*]	<i>C. aulicus</i>	750	ND	—	ND	14
RegIIA	GCCHHPACNVNPNPHIC [*]	<i>C. regius</i>	47.3	147	3.1	$\alpha 3\beta 2 > \alpha 3\beta 4 \approx \alpha 6\beta 2 > \alpha 7 > \alpha 6\beta 4$	20
[N11,12A]RegIIA	GCCHHPACNVVAAPHIC [*]	synthetic	370	5100	14	$\alpha 3\beta 4 > \alpha 6/\alpha 3\beta 4$	17
PeIA	GCCHHPACSVNHPHEL C [*]	<i>C. peregrandis</i>	480	1500	3.1	$\alpha 9\alpha 10 > \alpha 3\beta 2 > \alpha 6\beta 2 > \alpha 3\beta 4 > \alpha 7$	21
PIA	RDPCSNPVCVTHNPQIC	<i>C. purpurascens</i>	518	30	0.058	$\alpha 6/\alpha 3\beta 2 > \alpha 6/\alpha 3\beta 4 \approx \alpha 3\beta 2 > \alpha 3\beta 4$	13
BulIA	GCCHSTPPCAVLY—C [*]	<i>C. bullatus</i>	27.7	2.1	0.076	$\alpha 6/\alpha 3\beta 2 > \alpha 6/\alpha 3\beta 4 > \alpha 3\beta 2 > \alpha 3\beta 4$	22
[T5A, P60]BulIA	GCCHSAOPCAVLY—C [*]	synthetic	1200	58.1	0.048	$\alpha 6/\alpha 3\beta 4 > \alpha 3\beta 4$	18
TP-22/12-59	GCCHHPBCFBZY—C [*]	synthetic	2.3	ND	—	ND	19

^{*} Indicates a C-terminal amide.

^aRatio of $\alpha 6/\alpha 3\beta 4$: $\alpha 3\beta 4$ IC₅₀ values. B is 2-aminobutyric acid, Z is norvaline. ND, not determined.