An Unusual Pattern of Ligand-Receptor Interactions for the $\alpha 7$ Nicotinic Acetylcholine Receptor, with Implications for the Binding of Varenicline[§]

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ABSTRACT

The $\alpha 7$ nicotinic acetylcholine receptor shows broad pharmacology, complicating the development of subtype-specific nicotinic receptor agonists. Here we use unnatural amino acid mutagenesis to characterize binding to $\alpha 7$ by the smoking cessation drug varenicline (Chantix; Pfizer, Groton, CT), an $\alpha 4\beta 2$ -targeted agonist that shows full efficacy and modest potency at the $\alpha 7$ receptor. We find that unlike binding to its target receptor, varenicline does not form a cation- π interaction with TrpB, further supporting a unique binding mode for the cationic amine of nicotinic agonists at the $\alpha 7$ receptor. We also evaluate binding to the complementary face of the receptor's

binding site by varenicline, the endogenous agonist acetylcholine, and the potent nicotine analog epibatidine. Interestingly, we find no evidence for functionally important interactions involving backbone NH and CO groups thought to bind the canonical agonist hydrogen bond acceptor of the nicotinic pharmacophore, perhaps reflecting a lesser importance of this pharmacophore element for $\alpha 7$ binding. We also show that the Trp55 and Leu119 side chains of the binding site's complementary face are important for the binding of the larger agonists epibatidine and varenicline, but dispensable for binding of the smaller, endogenous agonist acetylcholine.

Introduction

The $\alpha 7$ nicotinic acetylcholine receptor (nAChR) is a principal mediator of synaptic transmission in the central nervous system. It has attracted considerable interest as a therapeutic target for Alzheimer's disease, schizophrenia, and inflammation (Martin et al., 2004; de Jonge and Ulloa, 2007; Hernandez and Dineley, 2012), and a number of $\alpha 7$ -directed compounds are currently in the clinic (Mazurov et al., 2011). A wide range of structural types activates the receptor (Horenstein et al., 2008), making drug development a serious challenge. Varenicline, which targets the $\alpha 4\beta 2$ nAChR and is currently prescribed for smoking cessation (Chantix; Pfizer, Groton, CT) (Coe et al., 2005), is a full agonist of $\alpha 7$ with modest potency (Mihalak et al., 2006). Adverse neuropsychiatric effects of Chantix suggest that varenicline could have off-target activity at $\alpha 7$ receptors (Papke et al., 2011b).

The $\alpha 7$ receptor has five identical agonist binding sites, located at each subunit-subunit interface. Crystal structures of snail acetylcholine binding proteins (AChBPs), homologous to the extracellular domain of nAChRs, provide a useful guide

for the binding site (Sixma and Smit, 2003; Rucktooa et al., 2009). Two chimeras of the $\alpha 7$ extracellular domain and an AChBP have been reported, one in complex with the antagonist methyllycaconitine (Nemecz and Taylor, 2011) and another in complex with the agonist epibatidine (Li et al., 2011) (Fig. 1A). Unmodified AChBP structures in complex with varenicline also suggest a ligand binding mode for this agonist (Billen et al., 2012; Rucktooa et al., 2012). As for other nAChRs, the $\alpha 7$ binding site features an "aromatic box" defined by four residues (TyrA, TrpB, TyrC1, and TyrC2) contributed by its "principal" face and one (TrpD) contributed by its "complementary" face on the adjacent subunit (Fig. 1) (Dougherty, 2008). This motif accommodates the positive charge common to all orthosteric nicotinic agonists (Beers and Reich, 1970).

The remainder of the binding site is contributed by the adjacent subunit. This complementary face recognizes the hydrogen bond acceptor moiety of the nicotinic pharmacophore. AChBP structures suggest that the hydrogen bonding partner is a water molecule held between the backbone NH of Leu119 and the backbone CO of Asn107 (Celie et al., 2004; Hansen et al., 2005; Talley et al., 2008; Rohde et al., 2012; Rucktooa et al., 2012). The proposed water molecule is not always evident in AChBP structures, but the positioning of key protein residues, including Leu119 and Asn107, is consistent in all structures, suggesting the water molecule is indeed present. As such, in Fig. 1 we added the proposed

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ABBREVIATIONS: 4-MeO-Phe, 4-methoxy-phenylalanine; ACh, acetylcholine; AChBP, acetylcholine binding protein; CN, cyano; F₃-Phe, 3,4,5-trifluoro-phenylalanine; nAChR, nicotinic acetylcholine receptor; hRIC-3, human resistance to inhibitors of cholinesterase 3; tRNA, transfer RNA.

Fig. 1. Two views of the nAChR agonist binding site. (A) Crystal structure of the *L. stagnalis* acetylcholine binding protein (*Ls*-AChBP)/ $\alpha 7$ chimera with epibatidine bound (3SQ6) (Li et al., 2011). The water molecule shown (red sphere) is not seen in this particular structure, but has been placed in the position seen in other structures. All side chains shown in this structure are identical to the actual receptor residues studied except for Leu107, which instead is Asn. (B) Schematic of the agonist binding site, showing the key noncovalent interactions considered here. Varenicline is shown as the agonist so that all interactions considered in the text can be illustrated. Solid purple lines: cation- π interactions; dashed red lines: hydrogen bonds. In both images, green residues are from the primary face; blue residues are from the complementary face.

hydrogen bonding water molecule to the AChBP structure. Previously, we used unnatural amino acid mutagenesis to establish that the backbone NH of Leu119 does have a hydrogen bonding interaction with agonists in the $\alpha 4\beta 2$ and muscle-type nAChRs, but the interaction is attenuated in the $\alpha 4\beta 4$ receptor (Blum et al., 2010). Similar evaluation of the Asn107 backbone CO in the muscle-type and $\alpha 4\beta 4$ receptors did not reveal a functionally important hydrogen bonding interaction (Blum et al., 2013).

Several side chains on the complementary face of the binding site are also positioned to form possible ligand contacts. The AChBP/varenicline structures suggest that side chains corresponding to $\alpha 7$ residues Trp55, Leu109, Gln117, and Leu119 contact the ligand (Fig. 1A) (Billen et al., 2012; Rucktooa et al., 2012). Both Trp55 and Leu119 have recently been implicated in ligand binding for the $\alpha 7$ receptor (Papke et al., 2011a).

In earlier work on $\alpha 7$, we showed that acetylcholine (ACh) and epibatidine make a cation- π interaction. (Puskas et al., 2011). Surprisingly, however, the residue involved was either TyrA, for ACh, or TyrC2, for epibatidine—all other nicotinic receptor/agonist combinations we have examined involve

a cation- π interaction to TrpB (Zhong et al., 1998; Xiu et al., 2009; Tavares et al., 2012). Additionally, a strong hydrogen bond from TrpB's backbone carbonyl to the agonist N⁺-H, which has been seen in the $\alpha 4\beta 2$ and $\alpha 4\beta 4$ neuronal receptors (Xiu et al., 2009; Puskar et al., 2011) and the muscle-type receptor (Cashin et al., 2005), is weak or absent for epibatidine at the $\alpha 7$ receptor.

Here we evaluate the binding of varenicline to the aromatic box of $\alpha 7$ and interactions to the complementary face for the agonists ACh, epibatidine, and varenicline (Fig. 2). We find a much different binding mode for varenicline at $\alpha 7$ than at the $\alpha 4\beta 2$ receptor, and the results for all the agonists confirm the unique binding behavior of $\alpha 7$ versus other nAChRs. We also identify residues on the complementary face that impact binding of epibatidine and varenicline but not of the smaller agonist ACh.

Materials and Methods

Molecular Biology. cDNA for the rat $\alpha 7$ T6'S receptor and for human resistance to inhibitors of cholinesterase 3 (hRIC-3) were in the pAMV and pGEM plasmids, respectively. Site-directed mutagenesis was performed using the QuikChange protocol (Agilent Technologies, Santa Clara, CA). For nonsense suppression experiments, the site of interest was mutated to the amber stop codon, with the exception of Val108 and Leu119, which were mutated to the opal stop codon. Circular $\alpha 7$ and hRIC-3 DNA were linearized with NotI and XhoI restriction enzymes, respectively. After purification (Qiagen, Hilden, Germany), linearized DNA was used as a template for runoff in vitro transcription using the T7 mMessage mMachine kit (Life Technologies, Carlsbad, CA). The amber suppressor transfer RNA (tRNA)^{THG73} (Saks et al., 1996) was used for nonsense suppression at all sites except Val108 and Leu119, for which the opal suppressor TQOpS' (Rodriguez et al., 2007a,b) was used.

α -Hydroxy or amino acids were appended to the dinucleotide dCA and enzymatically ligated to the appropriate truncated 74 mer suppressor tRNA as previously described (Nowak et al., 1998; England et al., 1999). Crude tRNA-amino acid or tRNA-hydroxy acid product was used without desalting, and the product was confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry on a 3-hydroxyphenylacetic acid matrix. tRNA-amino acids bearing a 6-nitroveratryloxycarbonyl protecting group were deprotected prior to injection via irradiation with a 500 W Hg/Xe arc lamp, filtered with WG-334 and UG-11 filters, prior to injection.

Microinjection. Stage V–VI *Xenopus laevis* oocytes were harvested and injected with RNAs as described previously (Nowak et al., 1998). Five to twenty-five nanograms of $\alpha 7$ mRNA was coinjected with ~20 ng of hRIC-3 mRNA per oocyte. For all of the suppression experiments, ~15 ng of tRNA per cell was used. Each oocyte was injected with 50 nl of RNA solution and incubated for 24–48 hours before recording. In the case of low maximal currents sometimes observed in nonsense suppression experiments, presumably because of low expression, a second RNA injection was required 24 hours after the first injection. As a negative control for suppression experiments, unacylated full-length tRNA was coinjected with mRNA in the same manner as charged tRNA. These control experiments yielded negligible responses for all sites studied.

Electrophysiology. Receptor function was assayed using the OpusXpress 6000A (Molecular Devices, Sunnyvale, CA) in two-electrode voltage clamp mode. The oocytes were clamped at a holding potential of -60 mV. One microliter of each drug solution was applied for 15 seconds, followed by a 5-minute wash step with ND96 buffer (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 5 mM HEPES) between each concentration. Acetylcholine chloride was purchased from Sigma-Aldrich (St. Louis, MO), ($\pm$)-epibatidine was purchased from Tocris (Bristol, UK), and varenicline tartrate was a generous gift

from Pfizer. Drug dilutions were prepared in ND96 buffer. Dose-response data were obtained for at least six concentrations of agonist and for a minimum of four oocytes. The EC_{50} and Hill coefficient values for each condition were obtained by fitting the averaged, normalized dose-response data to the Hill equation.

Results

Experimental Design. Recently, we showed that the $\alpha 7$ nAChR is amenable to unnatural amino acid mutagenesis by nonsense suppression in *Xenopus* oocytes—electrophysiology yields reproducible dose-response relationships when incorporating a wide panel of unnatural side chains (representative dose-response curves from this study are shown in Fig. 3) (Puskar et al., 2011). We coexpress the rat $\alpha 7$ nAChR with the hRIC-3 protein to overcome poor receptor expression. All mutant receptors studied include the T6'S background mutation in the transmembrane domain, which markedly reduces the effects of the rapid desensitization associated with the $\alpha 7$ receptor while minimally perturbing receptor pharmacology (Placzek et al., 2005).

In the present study we measure the functional impact of each mutation using EC_{50} , the effective agonist concentration that gives a half-maximal response. EC_{50} is a composite measure that reflects multiple equilibria: both “binding” events—drug entering/exiting the agonist binding site—and “gating” events—the equilibria between open and closed states of the channel. Without detailed kinetic analyses,

typically at the single channel level, it is not possible to parse which equilibria are perturbed by a given mutation. For example, a mutation affecting a binding interaction could affect a gating equilibrium if the drug binds more tightly to the open state of the channel than the closed state (or vice versa). The primary tool used here is unnatural amino acid mutagenesis, which allows precise, chemically well-defined modifications to the agonist binding site. We would argue that although the identity of the equilibrium step(s) being perturbed for each mutation in this study is unknown, we can confidently assign the nature of the perturbation: an attenuated ligand binding interaction. Our structural knowledge of the binding site has guided the location of mutations made, and previous studies on this and related systems demonstrate ligand-specific EC_{50} shifts consistent with specific binding interactions. Furthermore, unnatural amino acid mutagenesis allows for subtle and precise modifications to protein structure that target the interaction of interest. This argument is less compelling when more perturbing, conventional amino acid mutagenesis is employed, but studies of that kind reported here can provide guidance for further investigation.

We consider EC_{50} to be the appropriate metric here for two reasons. First, detailed, single channel studies are not feasible for the large number of drug-receptor combinations that we have considered. This is especially so given the protein expression limitations that are sometimes seen with unnatural amino acid mutagenesis. Second and more importantly, our goal is to evaluate the pharmacology of the $\alpha 7$ receptor and compare these results with those of previous studies on related systems. EC_{50} is a good measure of pharmacological activity. Given our experience with this system, we generally do not ascribe specific structural interactions to EC_{50} differences that are less than a factor of 2.

Unnatural Amino Acid Mutagenesis to Probe Cation- π Interactions and Hydrogen Bonds to the Protein Backbone. To determine whether varenicline forms a cation- π interaction to one or more of the aromatic residues on the binding site's principal face, we incorporated unnatural aromatic amino acid analogs with attenuated cation- π binding ability and probed for a concomitant reduction in receptor function (Fig. 2B). In particular, systematic fluorination of an aromatic side chain is diagnostic for a cation- π interaction. Comparing EC_{50} shifts for the highly deactivating cyano (CN) substituent and the sterically similar but much less deactivating bromo substituent is also instructive.

We probe hydrogen bonding to the protein backbone with α -hydroxy acid mutagenesis, replacing the native amide peptide bond with an ester (Fig. 2C). This mutation has two effects: most obviously, the hydrogen bond donor NH is deleted. Second, the ester carbonyl is well established as a poorer hydrogen bond acceptor than the native amide carbonyl, so hydrogen bonds to this group will be attenuated. Note that a backbone carbonyl is modulated by mutating the $i+1$ residue to an α -hydroxy acid.

Varenicline Interactions with the Binding Site's Principal Face. At the TrpB site, the varenicline EC_{50} for 5,6,7-trifluoro-tryptophan is not markedly shifted from wild type, whereas 4,5,6,7-tetrafluoro-tryptophan shows a modest 3.8-fold shift (Table 1), substantially smaller than we have observed for agonists forming a cation- π interaction with this side chain in other receptors. For comparison, a 16- to 20-fold

Fig. 2. (A) Agonists considered here. (B) Aromatic unnatural amino acids employed here. (C) The α -hydroxy acid strategy for evaluating backbone hydrogen bonding. The hydrogen bond is stronger (solid line) on the left than on the right (dashed line).

Fig. 3. Representative traces and dose-response curves. (A) Representative current traces for incorporation of Trp by nonsense suppression at the TrpB (Trp149) site. Bars indicate application of varenicline (in micromolar) at concentrations noted. (B) Dose-response curve and fit to the Hill equation for normalized varenicline responses for Trp incorporation at the TrpB site. Error bars indicate S.E.M. ($n = 17$).

shift was seen for the corresponding 4,5,6,7-tetrafluoro-tryptophan mutation with varenicline at its targeted $\alpha 4\beta 2$ receptor (Tavares et al., 2012). Furthermore, for $\alpha 7$ we found

TABLE 1

Mutations to the putative varenicline-binding residues of the binding site's principal face

Full data, including error estimates, are reported in Supplemental Table 1.

	Varenicline	
	EC ₅₀	Fold Shift
	μM	
Tyr93 (TyrA)		
Tyr ^a	2.2	
Phe	130	57
4-F ₁ -Phe	34	15
F ₂ -Phe	>100 ^b	
F ₃ -Phe	>100 ^b	
4-Br-Phe	12	5.4
4-CN-Phe	33	15
4-MeO-Phe	2.2	1.0
Trp149 (TrpB)		
Trp ^a	2.5	
F ₃ -Trp	4.1	1.6
F ₄ -Trp	9.6	3.8
4-CN-Trp	2.1	1/1.2
Tyr195 (TyrC2)		
Tyr ^a	2.1	
Phe	2.3	1.1
3-F ₁ -Phe	5.1	2.4
4-F ₁ -Phe	1.5	1/1.4
F ₂ -Phe	16	7.7
F ₃ -Phe	16	7.5
4-Br-Phe	3.5	1.6
4-CN-Phe	21	9.9
4-MeO-Phe	2.4	1.1
Ser150 (TrpB+1)		
Thr	0.81	1/2.5
Tah	2.4	3.0 from Thr

3-F₁-Phe, 3-fluoro-phenylalanine; 4-Br-Phe, 4-bromo-phenylalanine; 4-CN-Phe, 4-cyano-phenylalanine; 4-F₁-Phe, 4-fluoro-phenylalanine; F₂-Phe, 3,5-difluoro-phenylalanine; F₃-Trp, 5,6,7-trifluoro-tryptophan; F₄-Trp, 4,5,6,7-tetrafluoro-tryptophan; Tah, α -hydroxythreonine.

^a Expression of the wild-type receptor with the natural amino acid incorporated by nonsense suppression.

^b Response of naive oocytes to varenicline doses ≥ 1 mM obscures complete dose-response data.

that the highly deactivating cyano substituent has no functional effect, confirming that varenicline does not form a cation- π interaction to TrpB.

The remaining aromatic residues of the principal face, TyrA, TyrC1, and TyrC2, were also probed for cation- π binding to varenicline. Interrogation of tyrosine presents an additional challenge: fluorination of this aromatic system progressively lowers the pK_a of the hydroxyl group. This effect is substantial enough that tetrafluorotyrosine ($\text{pK}_a \sim 5.3$ versus 10 for tyrosine) will likely be deprotonated under physiologic conditions, confounding interpretation of EC₅₀ shifts (Thorson et al., 1995). We circumvent this complication by first evaluating the function of phenylalanine and then proceeding with fluorinated phenylalanine derivatives.

As we previously observed for ACh and epibatidine, phenylalanine produces a substantial EC₅₀ shift at TyrA for varenicline, whereas 4-methoxy-phenylalanine (4-MeO-Phe) produces a wild-type EC₅₀, possibly indicating the need for a hydrogen bond acceptor and/or steric placeholder at the 4-position (Table 1). 3,5-Difluoro-phenylalanine and 3,4,5-trifluoro-phenylalanine (F₃-Phe) gave small, but detectable, responses at high varenicline doses. Full dose-response curves, however, were obscured by the response of naive oocytes to varenicline at concentrations ≥ 1 mM. Without these data it is not possible to rule on a cation- π interaction at TyrA, although losses of function for 4-bromo-phenylalanine and more so for 4-cyano-phenylalanine do suggest an important role for side chain electrostatics (Supplemental Fig. 1).

In previous work on the $\alpha 7$ receptor, we assigned a cation- π interaction between ACh and TyrA (Puskas et al., 2011). However, a recent *Lymnaea stagnalis* acetylcholine binding protein/ $\alpha 7$ chimera crystal structure suggests that TyrA may actually form an intraprotein cation- π interaction with the Lys145 side chain (Fig. 1B) (Li et al., 2011). We neutralized this Lys side chain to Gln and found only a modest 4- to 6-fold loss of function for Var and ACh (Supplemental Table 1), suggesting that the much larger EC₅₀ shifts for poor cation- π -binding Phe analogs at TyrA largely reflect a weakened ligand binding interaction instead.

At TyrC2 the Phe mutant and 4-MeO-Phe are essentially wild type. We do observe a loss of receptor function for varenicline, with Phe analogs possessing attenuated cation- π binding ability (Table 1). However, EC₅₀ shifts are modest. For example, we see a 7.5-fold shift for the highly deactivating F₃-Phe mutation, whereas the same mutation for epibatidine (which does form a cation- π interaction with TyrC2) gave a nearly 50-fold shift (Puskar et al., 2011). Furthermore, the EC₅₀ values have only a weak linear dependence on cation- π binding energy (Supplemental Fig. 2). 4-Fluoro-phenylalanine unexpectedly gave a gain of receptor function, although shifting the fluorine to the 3-position produces a modest loss of function. TyrC1 was too sensitive to phenylalanine substitution to even yield a measurable EC₅₀, preventing the study of this residue (Supplemental Table 1).

Finally, we probed the TrpB backbone carbonyl for a functionally important hydrogen bond to the agonist, as has been observed in other receptors with agonists possessing an N⁺-H group. We find that mutation of Ser150 (*i* + 1 relative to TrpB) to α -hydroxythreonine produces a threefold shift relative to the conservative mutation S150Thr, potentially indicating a weak hydrogen bond (Table 1).

Probing the Canonical Hydrogen Bond of the Nicotinic Pharmacophore between Agonists and the Complementary Subunit. To test for the functional importance of interactions with the hydrogen bond acceptor group on the agonist, we evaluated residues that contribute to the water-mediated hydrogen bonding array of Fig. 1. The Leu119 backbone NH and the Asn107 backbone CO were evaluated by α -hydroxy acid mutagenesis. The L119 α -hydroxyisoleucine mutation, which deletes the Leu119 backbone NH, had little functional effect on the agonists ACh and epibatidine, with EC₅₀ shifts less than twofold, and produced a 2.6-fold shift for epibatidine (Table 2). We probed the Asn107 CO by mutating its *i* + 1 residue, Val108, to α -hydroxyvaline, attenuating the hydrogen bond accepting ability of the backbone CO of interest. We saw little functional effect for ACh and epibatidine, with EC₅₀ shifts less than twofold, and a modest 4.3-fold shift for varenicline.

Functional Importance of Side Chains on the Complementary Face of the Binding Site. To complete our survey of ligand-receptor interactions, we tested the functional effect of alanine mutations on residues of the binding site's complementary face that are proposed to contact these agonists: Trp55, Leu109, Gln117, and Leu119 (Li et al., 2011;

Billen et al., 2012; Rucktooa et al., 2012). Of these mutations, L109A and Q117A produced modest shifts of 3.1-fold or less for all three agonists tested (Table 3). Interestingly, the mutants W55A and L119A showed little, if any, effect on ACh, but large shifts of ninefold or greater for the larger agonists epibatidine and varenicline.

Discussion

This work expands our survey of ACh and epibatidine at the $\alpha 7$ nAChR and examines the yet-uncharacterized binding of varenicline. The results are directly comparable with studies of other nAChR subtypes, providing potential insights into the diverse pharmacologies of these receptors.

The "aromatic box" is conserved across all nicotinic binding sites. The present results for varenicline corroborate earlier findings for ACh and epibatidine: TrpB does not make a cation- π interaction (Puskar et al., 2011). This contrasts many studies of other nAChRs and other Cys-loop receptors, all of which involve cation- π interactions to an aromatic residue at the TrpB site (Zhong et al., 1998; Beene et al., 2002; Lummis et al., 2005; Pless et al., 2008; Xiu et al., 2009; Puskar et al., 2011; Tavares et al., 2012).

Instead, TyrA and TyrC2 of the $\alpha 7$ receptor form cation- π interactions with ACh and epibatidine, respectively (Puskar et al., 2011). For varenicline at $\alpha 7$, evaluation of TyrC2 produced a modest effect when substituting poor cation- π binding side chains and only a weak correlation of cation- π binding energy with EC₅₀ (Supplemental Fig. 2). We conclude that TyrC2 does not form a strong cation- π interaction with varenicline. At the TyrA site we do observe a suggestive electrostatic trend with varenicline (Supplemental Fig. 1), but we lack data for 3,5-difluoro-phenylalanine and F₃-Phe, hampered by EC₅₀ values beyond our measurable range. We also evaluated a proposed cation- π interaction between TyrA and Lys145. We find that K145Q produces an smaller effect than 4-F and 4-CN mutations of TyrA (Supplemental Table 1), which is not consistent with this proposal. For $\alpha 7$, as for other nAChRs, extreme sensitivity of TyrC1 to mutagenesis prevented further study of this residue.

We also evaluated several hydrogen bonding interactions in $\alpha 7$. It is important to note that we are probing the *functional* role of particular hydrogen bonds. Thus, it is possible that a structural study could show the presence of a hydrogen bond, but if deleting/attenuating that hydrogen bond has no functional consequence, it would show up as no hydrogen bond in our assay.

TABLE 2

Hydroxy acid mutations to putative backbone hydrogen bonding groups of the binding site's complementary face
EC₅₀ values (μ M) reported. Full data, including error estimates, are reported in Supplemental Table 2.

	Acetylcholine	Epibatidine	Varenicline
Probing Leu119 NH			
wt recovery (Leu) ^a	120	0.29	2.3
Mutant (Lah)	180	0.75	3.2
Fold shift	1.5	2.6	1.4
Probing Asn107 CO			
wt recovery (Val) ^a	100	0.40	2.3
Mutant (Vah)	180	0.64	10
Fold shift	1.8	1.6	4.3

Lah, α -hydroxyisoleucine; Vah, α -hydroxyvaline; wt, wild-type.

^a Expression of the wild-type receptor with the natural amino acid incorporated by nonsense suppression.

TABLE 3

Side chain mutations to putative ligand-contacting residues of the binding site's complementary face

Full data, including error estimates, are reported in Supplemental Table 2.

	Acetylcholine		Epibatidine		Varenicline	
	EC ₅₀	Fold Shift	EC ₅₀	Fold Shift	EC ₅₀	Fold Shift
	μ M		μ M		μ M	
wt	99		0.34		2.0	
W55A	130	1.4	5.8	17	68	34
L109A	300	3.1	0.28	1/1.2	0.53	1/3.8
Q117A	180	1.8	0.90	2.6	4.9	2.4
L119A	210	2.1	3.2	9.4	41	21

wt, wild-type.

We see only a weak hydrogen bond between the TrpB backbone carbonyl and the N⁺-H of varenicline (Table 1) (Puskar et al., 2011). The threefold EC₅₀ shift seen here was 14- to 19-fold for varenicline at its targeted $\alpha 4\beta 2$ receptor (Tavares et al., 2012) and was comparably larger for all nAChR/agonist pairs, as long as the agonist has the critical N⁺-H moiety (Xiu et al., 2009; Puskar et al., 2011; Tavares et al., 2012). Because all these other nAChR/agonist combinations involve a cation- π interaction to TrpB, it is perhaps not surprising that this hydrogen bond would be attenuated in $\alpha 7$ if the agonist has moved its cation- π interaction to other residues of the aromatic box.

AChBP crystal structures suggest a hydrogen bond between the side chain -OH of TyrA and the N⁺-H of agonists as shown in Fig. 1 (Celie et al., 2004; Hansen et al., 2005; Li et al., 2011; Rucktooa et al., 2012), and deleting the -OH by mutation to Phe results in a large loss of function (Table 1). Further supporting this view, 4-MeO-Phe, which can serve as a hydrogen bond acceptor like Tyr, shows wild-type behavior. 4-F₁-, 4-CN-, and 4-bromo-phenylalanine are all preferable to Phe, suggesting there could also be a steric component involving the 4-position of TyrA.

At TyrC2, mutation to Phe yields a receptor with wild-type function for varenicline (Table 1). AChBP-varenicline crystal structures show a potential hydrogen bond between the TyrC2 -OH and varenicline's quinoxaline nitrogens (Billen et al., 2012; Rucktooa et al., 2012). Our data indicate that any such interaction in the $\alpha 7$ receptor is not functionally important. Interestingly, the Phe mutant did have a markedly shifted EC₅₀ for ACh and for epibatidine (Puskar et al., 2011), so varenicline interacts with this side chain differently than other agonists. Other mutations at TyrC2 do not strongly impact varenicline function.

The classic nicotinic pharmacophore includes a hydrogen bond acceptor approximately 4-6 Å away from the cationic group (Beers and Reich, 1970). AChBP crystal structures implicate a water molecule that is held in place by a backbone NH and backbone CO of the complementary subunit (Fig. 1). Backbone mutagenesis and mutant cycle analyses established an important hydrogen bonding role for the Leu119 backbone NH in the $\alpha 4\beta 2$ and muscle-type nAChRs but a lesser role in $\alpha 4\beta 4$ (Blum et al., 2010, 2013). We now find that the appropriate backbone mutation of Leu119 in the $\alpha 7$ receptor has minimal functional effect on ACh and varenicline and only a 2.6-fold shift for epibatidine (Table 2). Evidently, the Leu119 NH does not form a functionally important interaction in the $\alpha 7$ receptor, perhaps reflecting either a reshaping of the binding site or a repositioning of agonists relative to their binding mode in the other receptors. A subtly different ligand binding mode might be expected, given the use of TyrA and TyrC2 for cation- π interactions in $\alpha 7$, rather than TrpB.

The backbone CO of Asn107 has also been proposed to contribute to the binding of agonists (Fig. 1). We find that, in the $\alpha 7$ receptor, ACh and epibatidine are largely insensitive to the V108 α -hydroxyvaline mutation that attenuates the hydrogen bond acceptor ability of the Asn107 CO (Table 2). We see a modest, 4.3-fold effect for varenicline. Analogous hydroxy acid mutations modulating this CO in the muscle-type and $\alpha 4\beta 4$ receptors did not affect agonist EC₅₀ (Blum et al., 2013). Note that α -hydroxy acid mutations probing backbone CO hydrogen bonding can produce large EC₅₀ shifts, an example being the TrpB backbone CO, where we have seen

shifts as large as 20- to 30-fold in other receptors (Xiu et al., 2009; Tavares et al., 2012).

Taken together, it appears that the $\alpha 7$ receptor either engages the agonist hydrogen bond acceptor with other groups or lacks energetically important contacts with it. Other candidate hydrogen bonding partners include the Gln117 and Asn107 side chains. However, mutation of these side chains has only modest functional effects of fourfold or smaller, suggesting that no critical interactions are present (Supplemental Table 2; Table 3).

Regarding the possibility that the agonist hydrogen bond acceptor is not functionally important in $\alpha 7$, tetramethylammonium, a structure that cannot participate in the hydrogen bonding interactions being probed here, has virtually the same potency and efficacy as ACh for the $\alpha 7$ receptor (Papke et al., 1996; Horenstein et al., 2008). Other nAChRs generally show substantially elevated EC₅₀ values and/or reduced efficacies with tetramethylammonium (Papke et al., 1996; Horenstein et al., 2008). It thus appears that the minimal requirements for agonist binding are more relaxed for $\alpha 7$. The homomeric $\alpha 7$ receptor is phylogenetically more ancestral than the subunits of heteromeric receptors (Le Novère et al., 2002) and appears to have a less specialized binding site with broad pharmacology (Horenstein et al., 2008). Indeed, some $\alpha 7$ -specific agonists lack the hydrogen bond acceptor probed here (Horenstein et al., 2008).

With the cationic moiety of agonists buried within the aromatic box, the remaining features of larger ligands might be expected to make important receptor contacts to the complementary face of the binding site, and crystal structures of varenicline bound to AChBP suggest several possibilities (Fig. 1) (Billen et al., 2012; Rucktooa et al., 2012). We find that ACh is minimally perturbed by alanine mutations to each of these residues (Table 3). The largest loss of function for ACh is observed for L109A (3.1-fold), which interestingly caused a modest gain of function (3.8-fold) for the larger varenicline ligand and a wild-type EC₅₀ for epibatidine. Steric compensation between the ligand and side chain could explain this observation. The Q117A mutation had only a small effect on all three agonists. The W55A and L119A mutations, however, were highly detrimental to epibatidine and varenicline, but unperturbing to ACh. These residues, more highly conserved than Leu109 and Gln117 among subunits forming the complementary face of nicotinic binding sites, could form hydrophobic interactions with larger ligands, but do not affect ACh.

The Trp55 and Leu119 side chains play an important functional role in the $\alpha 7$ receptor, with modifications selectively affecting larger agonists (Papke et al., 2011a). Strikingly, the W55A mutation has only a small effect on EC₅₀ for varenicline but increases the relative efficacy from 15 to 125% in the $\alpha 4\beta 2$ receptor, this drug's intended target (Billen et al., 2012). The substantial increase in EC₅₀ we observe for W55A in the $\alpha 7$ receptor represents a dramatically different phenotype and suggests that this residue makes different contributions to receptor function for $\alpha 7$ versus $\alpha 4\beta 2$, perhaps providing a strategy for improving $\alpha 4\beta 2$ selectivity over $\alpha 7$.

We found a unique pattern of agonist interactions in the $\alpha 7$ receptor compared with other nAChRs. Differing roles for conserved binding site residues across nAChRs underscore the challenge of rationalizing subtype selectivity and the critical importance of functional evaluation of interactions

suggested by structural models. Despite these challenges, the growing body of knowledge on subtype-specific drug-receptor interactions holds promise for advancing selectivity in drug design.

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Authorship Contributions

Participated in research design: Van Arnam, Blythe, Lester, Dougherty.

Conducted experiments: Van Arnam, Blythe.

Performed data analysis: Van Arnam, Blythe, Lester, Dougherty.

Wrote or contributed to the writing of the manuscript: Van Arnam, Dougherty.

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