Chapter 5: An unusual pattern of ligand-receptor interactions for the α7 nicotinic acetylcholine receptor, with implications for the binding of varenicline*

5.1 Abstract

The α7 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 α7 by the smoking cessation drug varenicline (Chantix), an α4β2-targeted agonist that shows full efficacy and modest potency at the α7 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 α7 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 significant 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 α7 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.

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5.2 Introduction

The α7 nicotinic acetylcholine receptor (nAChR), a member of the Cys loop (pentameric) family of ligand-gated ion channels, is one of the principal mediators of synaptic transmission in the central nervous system. It has attracted significant interest as a therapeutic target for Alzheimer's disease, schizophrenia, and inflammation,[1-3] and a number of α7-directed compounds are currently in the clinic for treatment of these disorders.[4]

Pharmacology of the α7 nAChR has revealed a wide range of structures capable of activating the receptor,[5] contributing to the challenge of advancing selectivity among receptor subtypes in CNS drug development. Varenicline, a potent partial agonist of the α4β2 receptor currently prescribed as a smoking cessation therapy (Chantix),[6] has also to been demonstrated to be a full agonist of α7 with modest potency.[7] Adverse neuropsychiatric effects of this drug have led to speculation that varenicline therapy could have off-target activity on α7 receptors.[8]

Despite a large and growing body of pharmacological data, our knowledge of the functionally important ligand-receptor interactions of the α7-binding site remains limited. The receptor has five identical agonist binding sites, located at each subunit-subunit interface. While no direct structural data yet exist for this receptor, a large number of crystal structures of snail acetylcholine binding proteins (AChBPs), homologous to the extracellular domain of nAChRs, provide a useful guide for the binding site. [9,10] Two chimeras of the α7 extracellular domain and an AChBP have recently been reported, one with the *Aplysia californica* AChBP in complex with the antagonist MLA[11] and another

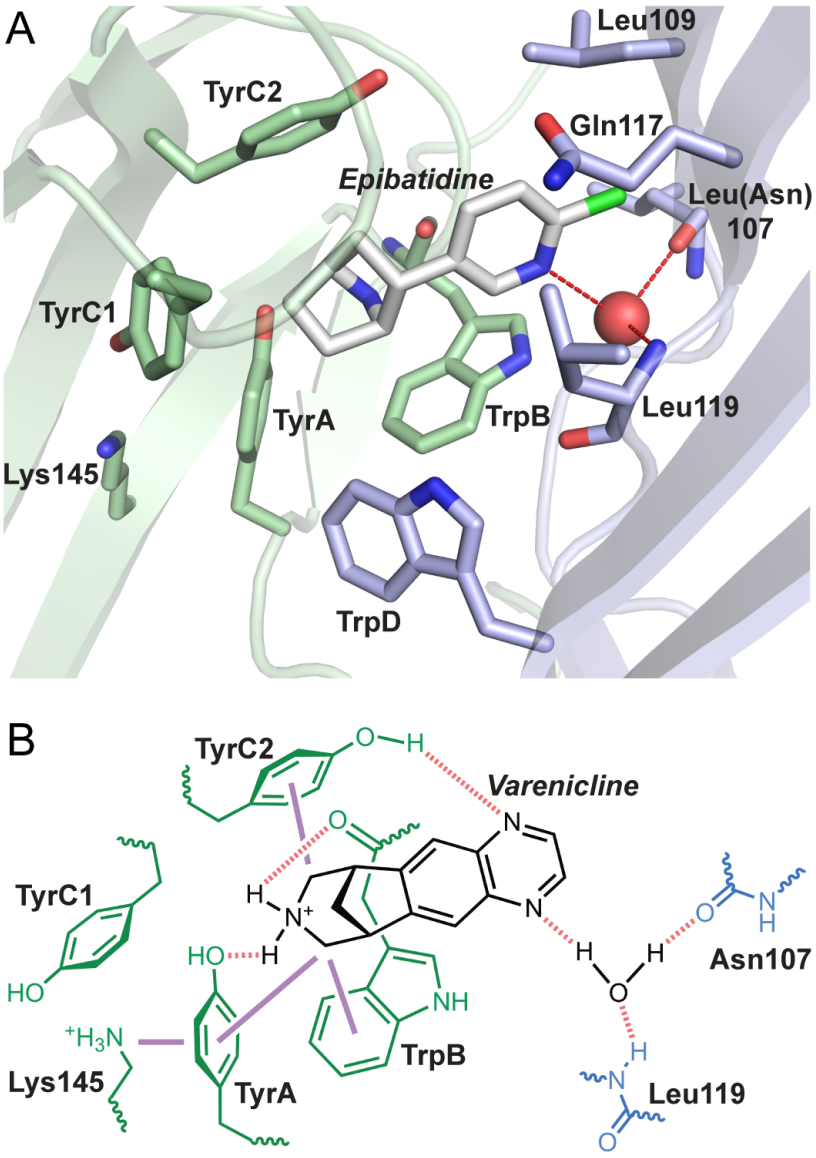

Figure 5.1. Two views of the nAChR agonist binding site. (A) Crystal structure of the *Ls*-AChBP/α7 chimera with epibatidine bound (3SQ6).[12] The water molecule shown (red sphere) is not seen in this particular structure, but has been placed in the position seen in other structures. All side chains 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 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.

with the *Lymnaea stagnalis* AChBP (*Ls*-AChBP) in complex with the agonist epibatidine.[12] Including all native α7 binding site residues and a bound agonist, the *Ls*-AChBP/α7 chimera provides an excellent starting place to identify important agonist-receptor interactions (Figure 5.1A). Recent unmodified AChBP structures in complex with varenicline also suggest a ligand binding mode for this agonist.[13,14] As for all other nicotinic receptors, the chief feature of the α7 binding site is 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 (Figure 5.1).[15] This motif accommodates the positive charge common to all orthosteric nicotinic agonists, the principal component of the classical nicotinic pharmacophore.[16]

The remainder of the binding site is contributed by the adjacent subunit. This complementary face of the binding site is thought to recognize the hydrogen bond acceptor moiety of the classical nicotinic pharmacophore. Various AChBP structures suggest that this hydrogen bonding partner is a water molecule held between the backbone NH of Leu119 and the backbone CO of Asn107.[14,17-20] The proposed water molecule is not always evident in the structures, but the positioning of the key protein residues, including Leu119 and Asn107, is consistent in all structures, and so the water molecule is assumed to be present. As such, in Figure 5.1 we have added the proposed hydrogen bonding water molecule to the *Ls*-AChBP/α7 chimera structure. In previous work, we have used unnatural amino acid mutagenesis to establish that the backbone NH of Leu119 does have a hydrogen bonding interaction with agonists in the α4β2 and muscle-type nicotinic receptors, but the interaction is attenuated in the α4β4 receptor.[21]

In contrast, similar evaluation of the Asn107 backbone CO in the muscle-type and α4β4 receptors did not reveal a functionally significant hydrogen bonding interaction.[22]

Several side chains on the complementary face of the binding site are also positioned to form possible ligand contacts. Recent crystal structures of AChBP in complex with varenicline have indicated that side chains corresponding to α7 residues Trp55, Leu109, Gln117, and Leu119 all contact this ligand (Figure 5.1A).[13,14] Both the Trp55 and Leu119 side chains have recently been implicated in ligand binding for the α7 receptor.[23]

We have previously characterized a unique binding mode for the endogenous agonist ACh and the potent nicotine analog epibatidine to the aromatic box residues of the α7 receptor.[24] As expected, the cationic moiety of the agonist binds *via* a cation-π interaction. Surprisingly, however, the residue involved was found to be either TyrA, for ACh, or TyrC2, for epibatidine – all other nicotinic receptor/agonist combinations we have examined involve a cation-π interaction to TrpB.[25-27] Additionally, a strong hydrogen bond from TrpB's backbone carbonyl to the agonist N^{+}-H (for agonists possessing this moiety), which has been seen in the α4β2 and α4β4 neuronal receptors [24,26] and the muscle-type receptor,[28] appears to be weak or absent for epibatidine at the α7 receptor.

In the present work, we use unnatural amino acid mutagenesis to evaluate binding of the fully efficacious and moderately potent agonist varenicline to the aromatic box residues of the α7 receptor's principal face. We also evaluate interactions to the receptor's complementary face for the agonists ACh, epibatidine, and varenicline. Like

ACh and epibatidine, varenicline does not form a cation-π interaction to TrpB. This observation further supports a unique binding mode to the aromatic box for agonists in α7. We also find that these three agonists are largely insensitive to backbone mutations to both the NH and CO groups thought to recognize the hydrogen bond acceptor of the nicotinic pharmacophore. By conventional mutagenesis of complementary face residues proposed to contribute to the binding site, we have identified side chains that are functionally important for the agonists epibatidine and varenicline, but not for the smaller agonist ACh.

5.3 Results

5.3.1 Experimental design

Recently, we have shown that the α7 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 Figure 5.2).[24] We coexpress the rat α7 nAChR with the human RIC-3 protein to overcome poor receptor expression. All mutant receptors studied include the T6'S background mutation in the transmembrane domain, which limits the rapid desensitization associated with the α7 receptor while minimally perturbing receptor pharmacology.[29]

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

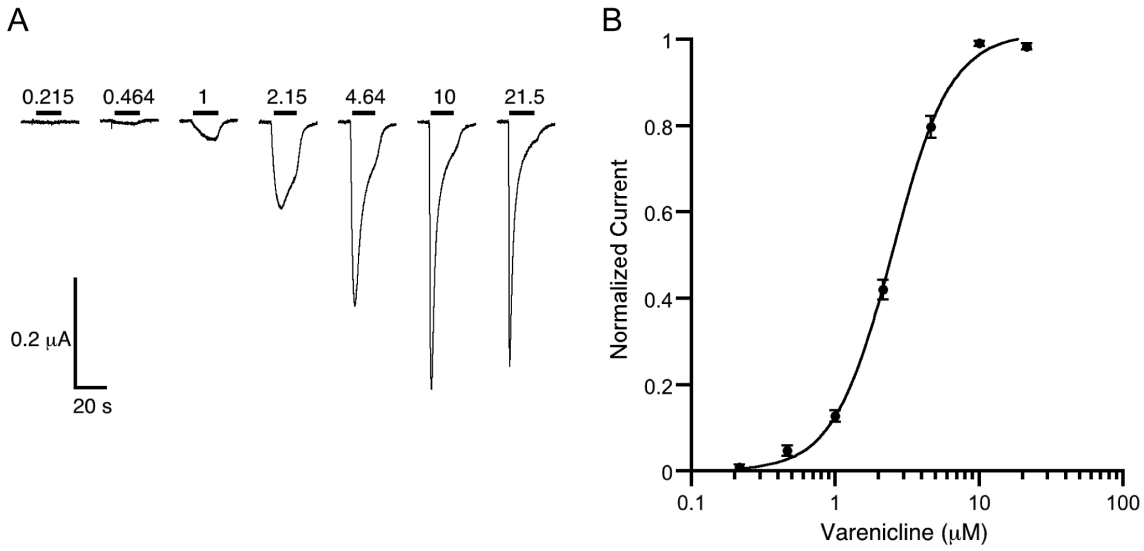

Figure 5.2. Representative traces and dose-response curves. (A) Representative current traces for incorporation of Trp by nonsense suppression at the TrpB (Trp149) site. Bars indicate application of varenicline (in μM) at concentrations noted. (B) Dose-response curve and fit to the Hill equation for normalized varenicline responses for Trp incorporation at the TrpB site. Error bars indicate SEM (n = 17).

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 while 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. Further, 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 α7 receptor and compare these results to 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.

5.3.2 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 (Figure 5.3B). 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 (Br) 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 (Figure 5.3C). 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.

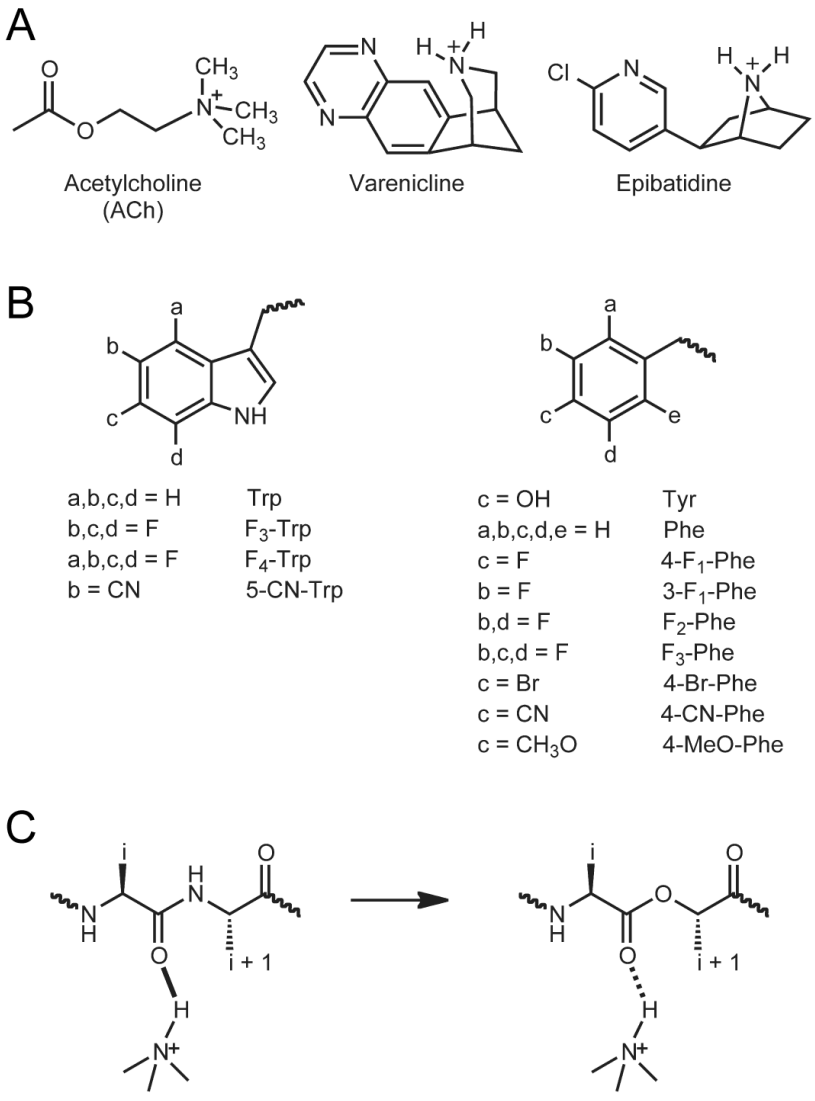

Figure 5.3. (A) Agonists considered here. (B) Aromatic unnatural amino acids employed here. (C) The α-hydroxy acid strategy for evaluating backbone hydrogen bonding. The hydrogen bond is stronger (solid line) on the left than on the right (dashed line).

5.3.3 Varenicline interactions with the binding site's principal face

At the TrpB site, the varenicline EC_{50} for F_3-Trp is not significantly shifted from wild type, while F_4-Trp shows a modest 3.8-fold shift (Table 5.1), substantially smaller than we have observed for agonists forming a cation-π interaction with this side chain in other receptors. For comparison, a 16-20-fold shift was seen for the corresponding F_4-Trp mutation with varenicline at its targeted α4β2 receptor.[25] Further, for α7 we found 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 (pK_a ~5.3 vs 10 for tyrosine) will likely be deprotonated under physiological conditions, confounding interpretation of EC_{50} shifts.[30] We circumvent this complication by first evaluating the function of phenylalanine and then proceeding with fluorinated phenylalanine derivatives.

As we had previously observed for ACh and epibatidine, phenylalanine produces a substantial EC_{50} shift at TyrA for varenicline, while 4-MeO-Phe produces a wild-type EC_{50}, possibly indicating the need for a hydrogen bond acceptor and/or steric placeholder at the 4-position (Table 5.1). F_2-Phe and F_3-Phe gave small, but detectable, responses at high varenicline doses. Full dose-response curves, however, were obscured by the response of naïve 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-

Br-Phe and more so for 4-CN-Phe do suggest an important role for side chain electrostatics (Figure 5.4).

Table 5.1. Wild type and mutations to the binding site's principal face. EC_{50} and Hill coefficient (n_H) are ± SEM for goodness of fit to the Hill equation.

Mutation	Agonist	EC_{50} (μM)	Fold Shift	n_H	n
wt	ACh	99 ± 3		2.7 ± 0.2	16
wt	Varenicline	1.99 ± 0.03		2.8 ± 0.1	15
wt	Epibatidine	0.34 ± 0.01		3.0 ± 0.2	13
TyrA					
Y93 Tyr[a]	Varenicline	2.21 ± 0.05		3.0 ± 0.2	7
Y93 Phe	Varenicline	126 ± 5	57	2.4 ± 0.2	9
Y93 4-F_1-Phe	Varenicline	34 ± 3	15	2.0 ± 0.3	11
Y93 F_2-Phe	Varenicline	>100[b]			8
Y93 F_3-Phe	Varenicline	>100[b]			8
Y93 4-Br-Phe	Varenicline	12 ± 1	5.4	3.1 ± 0.6	7
Y93 4-CN-Phe	Varenicline	33 ± 2	15	2.5 ± 0.4	10
Y93 4-MeO-Phe	Varenicline	2.19 ± 0.05	0.99	2.3 ± 0.1	6
TrpB					
W149 Trp[a]	Varenicline	2.5 ± 0.1		2.1 ± 0.1	17
W149 F_3-Trp	Varenicline	4.1 ± 0.1	1.6	2.3 ± 0.1	15
W149 F_4-Trp	Varenicline	9.6 ± 0.8	3.8	2.0 ± 0.2	15
W149 5-CN-Trp	Varenicline	2.1 ± 0.1	0.84	2.6 ± 0.4	12
TyrC1					
Y188 Tyr[a]	Varenicline	2.23 ± 0.08		2.4 ± 0.2	8
Y188 Phe	Varenicline	>100[b]			6
TyrC2					
Y195 Tyr[a]	Varenicline	2.12 ± 0.02		2.76 ± 0.06	8
Y195 Phe	Varenicline	2.28 ± 0.09	1.1	3.1 ± 0.4	6
Y195 3-F_1-Phe	Varenicline	5.1 ± 0.07	2.4	1.9 ± 0.02	12
Y195 4-F_1-Phe	Varenicline	1.53 ± 0.05	0.72	3.4 ± 0.3	9
Y195 F_2-Phe	Varenicline	16.3 ± 0.7	7.7	2.4 ± 0.2	12
Y195 F_3-Phe	Varenicline	16 ± 1	7.5	2.6 ± 0.3	7
Y195 4-Br-Phe	Varenicline	3.48 ± 0.07	1.6	3.6 ± 0.2	5
Y195 4-CN-Phe	Varenicline	21 ± 1	9.9	3.8 ± 0.9	11
Y195 4-MeO-Phe	Varenicline	2.42 ± 0.09	1.1	2.8 ± 0.3	11
TrpB+1					
S150 Thr	Varenicline	0.81 ± 0.04		2.2 ± 0.2	11
S150 Tah	Varenicline	2.4 ± 0.1	3.0 from Thr	2.2 ± 0.2	15
Lys145					
K145A	ACh	N.R.			
K145Q	ACh	590 ± 20	6.0	2.5 ± 0.1	8
K145Q	Varenicline	9.4 ± 0.2	4.7	1.6 ± 0.2	7
K145R	ACh	1600 ± 100	16	2.6 ± 0.3	6
K145R	Varenicline	61.6 ± 0.2	31	3.19 ± 0.03	9

[a] *Expression of the wild type receptor with the natural amino acid incorporated by nonsense suppression*
[b] *Response of naïve oocytes to varenicline doses ≥ 1mM obscures complete dose-response data*

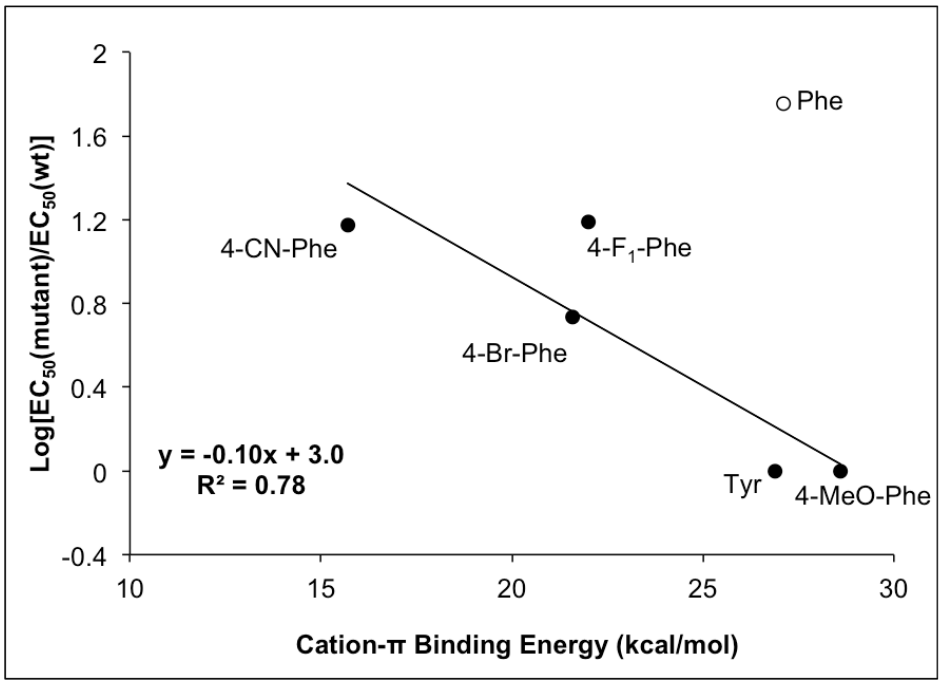

Figure 5.4. Cation-π binding plot for TyrA, in which log[EC_{50}(mutant)/EC_{50}(wt)] is plotted *versus* quantitative cation-π binding energies;[27,31] a strong linear correlation would suggest a cation-π interaction. Phe (open circle) is not included in the fit.

In previous work on the α7 receptor, we assigned a cation-π interaction between ACh and TyrA.[24] However, a recent *Ls*-AChBP/α7 chimera crystal structure suggests that TyrA may actually form an intra-protein cation-π interaction with the Lys145 side chain (Figure 5.1B).[12] We neutralized this Lys side chain to Gln and found only a modest 4-6-fold loss of function for Var and ACh (Table 5.1), suggesting that the much larger EC_{50} 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 5.1). However, EC_{50} shifts are modest. For example, we see a 7.5-fold shift for the highly deactivating F_3-Phe mutation, while the same mutation for epibatidine (which does form a cation-π interaction with TyrC2) gave a nearly 50-fold shift.[24] Further, the EC_{50}s have only a weak linear dependence on cation-π binding energy (Figure 5.5). 4-F_1-Phe unexpectedly gave a gain of receptor function, though 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 EC_{50}, preventing the study of this residue (Table 5.1).

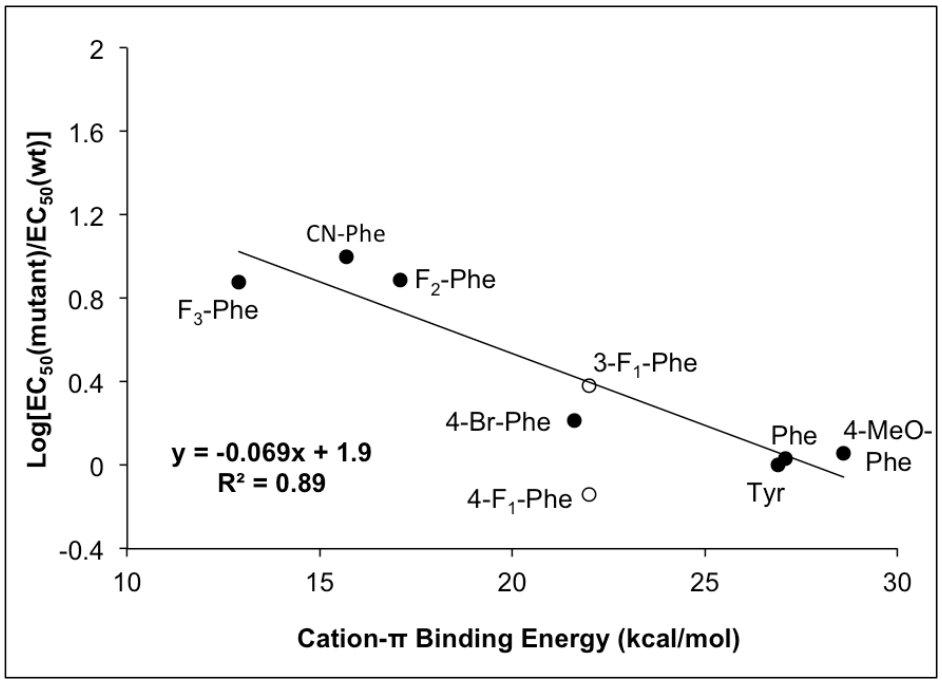

Figure 5.5. Cation-π binding plot for TyrA, in which log[EC_{50}(mutant)/EC_{50}(wt)] is plotted *versus* quantitative cation-π binding energies;[27,31] a strong linear correlation would suggest a cation-π interaction. 3-F_1-Phe and 4-F_1-Phe (open circles) are not included in the fit.

Finally, we probed the TrpB backbone carbonyl for a functionally significant 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 threonine, α-hydroxy (Tah) produces a three-fold shift relative to the conservative mutation S150Thr, potentially indicating a weak hydrogen bond (Table 5.1).

5.3.4 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 Figure 5.1. The Leu119 backbone NH and the Asn107 backbone CO were evaluated by α-hydroxy acid mutagenesis. The L119Lah mutation, which deletes the Leu119 backbone NH, had little functional effect on the agonists ACh and varenicline, with EC_{50} shifts less than 2-fold, and produced a 2.6-fold shift for epibatidine (Table 5.2). We probed the Asn107 CO by mutating its *i*+1 residue, Val108, to valine, α-hydroxy (Vah), attenuating the hydrogen bond accepting ability of the backbone CO of interest. We saw little functional effect for ACh and epibatidine, with EC_{50} shifts less than 2-fold, and a modest 4.3-fold shift for varenicline.

5.3.5 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.[12-14] Of these mutations, L109A and Q117A produced modest shifts of 3.1-fold or less for all three agonists tested (Table 5.2). Interestingly, the mutants W55A and L119A showed little, if

any, effect on ACh, but large shifts of 9-fold or greater for the larger agonists epibatidine and varenicline.

Table 5.2. Mutations to the binding site's complementary face. EC_{50} and Hill coefficient (n_H) are ± SEM for goodness of fit to the Hill equation.

Mutation	Agonist	EC_{50} (μM)	Fold Shift	n_H	n
V108 Val[a]	ACh	103 ± 2		2.9 ± 0.2	10
V108 Vah	ACh	184 ± 9	1.8	2.3 ± 0.2	10
V108 Val[a]	Varenicline	2.3 ± 0.1		4 ± 1	8
V108 Vah	Varenicline	10.0 ± 0.5	4.3	2.5 ± 0.3	9
V108 Val[a]	Epibatidine	0.396 ± 0.005		3.02 ± 0.09	7
V108 Vah	Epibatidine	0.64 ± 0.04	1.6	3.4 ± 0.5	11
L119 Leu[a]	ACh	120 ± 6		2.5 ± 0.3	9
L119 Lah	ACh	180 ± 8	1.5	2.4 ± 0.2	6
L119 Leu[a]	Varenicline	2.26 ± 0.02		2.76 ± 0.05	7
L119 Lah	Varenicline	3.15 ± 0.08	1.4	2.2 ± 0.1	9
L119 Leu[a]	Epibatidine	0.290 ± 0.005		3.3 ± 0.1	8
L119 Lah	Epibatidine	0.75 ± 0.01	2.6	3.4 ± 0.1	10
N107L	ACh	350 ± 3	3.5	2.48 ± 0.05	5
N107L	Varenicline	2.77 ± 0.05	1.4	2.6 ± 0.1	4
N107L	Epibatidine	1.37 ± 0.02	4.0	2.44 ± 0.06	4
W55A	ACh	134 ± 8	1.4	1.9 ± 0.2	8
W55A	Varenicline	67.6 ± 0.9	34	2.95 ± 0.09	11
W55A	Epibatidine	5.8 ± 0.3	17	2.2 ± 0.2	10
L109A	ACh	303 ± 7	3.1	2.4 ± 0.1	9
L109A	Varenicline	0.53 ± 0.01	0.27	3.3 ± 0.2	12
L109A	Epibatidine	0.282 ± 0.008	0.83	2.8 ± 0.2	9
Q117A	ACh	180 ± 4	1.8	2.7 ± 0.1	10
Q117A	Varenicline	4.85 ± 0.06	2.4	2.88 ± 0.09	12
Q117A	Epibatidine	0.90 ± 0.05	2.6	3.0 ± 0.4	11
L119A	ACh	210 ± 9	2.1	2.3 ± 0.2	11
L119A	Varenicline	41 ± 1	21	2.6 ± 0.2	11
L119A	Epibatidine	3.20 ± 0.09	9	3.0 ± 0.2	10

[a]*Expression of the wild type receptor with the natural amino acid incorporated by nonsense suppression*
[b]*Response of naïve oocytes to varenicline doses ≥ 1mM obscures complete dose-response data*

5.4 Discussion

This work expands our survey of ligand-receptor interactions for ACh and epibatidine at the α7 nAChR and examines the yet-uncharacterized binding of varenicline. We have previously investigated binding of these ligands to other nAChRs, allowing for

comparisons to be drawn among these receptors with superficially similar binding sites, but distinct pharmacologies.

5.4.1 Cation-π interactions to the "aromatic box" residues of the principal face

The "aromatic box" is conserved across all nicotinic binding sites and is comprised of identical residues: three tyrosines (A, C1, and C2) and two tryptophans (B and D). The present study of varenicline's interactions with these side chains corroborates our earlier findings for the binding of ACh and epibatidine to the α7 receptor: TrpB is not engaged in a cation-π interaction with the agonist.[24] This contrasts a large number of studies of other nAChRs, including the muscle-type receptor, the α4β4 receptor, and both stoichiometries of the α4β2 receptor, as well as other Cys-loop receptors such as the 5-HT_{3A} receptor, the glycine receptor, and the GABAc receptor, all of which involve cation-π interactions to TrpB or another aromatic residue at that site.[24-27,31-33]

Instead, TyrA and TyrC2 of the α7 receptor form cation-π interactions with the agonists ACh and epibatidine, respectively.[24] We were able to evaluate a number of TyrC2 mutants. We find a modest effect when substituting poor cation-π binding side chains and only a weak correlation of cation-π binding energy with EC_{50} (Figure 5.5). 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 for the Phe analogs incorporated (Figure 5.4), but we lack data for F_2-Phe and F_3-Phe, hampered by EC_{50} values beyond our measurable range. On the basis of the *Ls*-AChBP/α7 chimera structure a cation-π interaction between TyrA and Lys145 has been proposed. However, we find that the K145Q produces a smaller effect than the 4-F and 4-CN mutations of

TyrA (Table 5.1), which is not consistent with this model. For α7, as for other nicotinic receptors we have investigated, extreme sensitivity of TyrC1 to mutagenesis has prevented further study of this residue.

5.4.2 Hydrogen bonding and steric effects on the principal face

It is worth emphasizing that, while we consider the present work to probe hydrogen bonding interactions, we are in fact probing the *functional significance* 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.

We find evidence for only a weak hydrogen bond between the TrpB backbone carbonyl and the N^+-H of varenicline, as we had observed for epibatidine (Table 5.1).[24] The hydroxy acid mutation that here produced a 3-fold EC_{50} shift gave much larger 14-19-fold shifts for varenicline at its targeted α4β2 receptor.[25] Indeed, comparably large shifts have been measured for all agonists bearing this N^+-H that we have characterized to date at the α4β2 and α4β4 neuronal receptors, all of which also bind the TrpB side chain with a cation-π interaction.[24-26] It is perhaps not surprising, then, that this hydrogen bond would be attenuated if the agonist has moved its cation-π interaction to other residues of the aromatic box.

Various AChBP crystal structures suggest a hydrogen bond between the side chain –OH of TyrA and the N^+-H of agonists such as nicotine (1UW6), varenicline (4AFT), cytisine (4AFO), and epibatidine (2BYQ, 3SQ6), as shown in Figure 5.1.[12,14,17,18] The large loss of function for deleting the –OH by mutation to Phe is

consistent with this model (Table 5.1). In addition, 4-MeO-Phe, which can serve as a hydrogen bond acceptor like Tyr, shows wild type behavior. 4-F_1-, 4-CN-, and 4-Br-Phe are all preferable to Phe, suggesting there could also be a steric component involving the 4 position of TyrA.

For TyrC2, mutation to Phe yields a receptor with wild type function for varenicline (Table 5.1): neither a steric nor a more specific hydrogen bonding role for this side chain –OH seems plausible. Recent AChBP-varenicline crystal structures show a potential hydrogen bond between the TyrC2 –OH and one of varenicline's quinoxaline nitrogens.[13,14] Our data indicate that any such interaction in the α7 receptor is not functionally important. Interestingly, the Phe mutant did have a significantly shifted EC_{50} for ACh and for epibatidine (6-fold and 11-fold, respectively),[24] so varenicline evidently interacts with this side chain differently than these other agonists. Generally, mutations at this site do not strongly impact varenicline function, and there are no obvious trends in the data.

5.4.3 Hydrogen bonding to the complementary face

In addition to a cationic group, the classical nicotinic pharmacophore includes a hydrogen bond acceptor approximately 4-6 Å away,[16] a feature shared by all three agonists tested in this study. AChBP crystal structures show agonist hydrogen bond acceptor groups directly contacting a water molecule, which in turn is held by a backbone NH and backbone CO of the complementary subunit (Figure 5.1). Mutant cycle analyses of the α4β2 receptor confirm a hydrogen bonding role for the Leu119 backbone NH for both nicotine and ACh as agonists, consistent with the AChBP structures. In α4β2, the L119Lah mutant (which removes this residue's backbone NH) produced a 7-fold EC_{50}

shift for ACh and for nicotine.[21] We now find that the corresponding backbone mutation of Leu119 in the α7 receptor has minimal functional effects on ACh and varenicline and only a 2.6-fold shift for epibatidine (Table 5.2). Evidently, the Leu119 NH does not form a functionally important interaction in the α7 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 α7, rather than TrpB. Functional importance of the Leu119 NH in other nicotinic receptors is mixed: it forms important contacts to ACh and nicotine for the muscle-type nAChR, but significantly weaker interactions in the neuronal α4β4 receptor.[22]

The other proposed hydrogen bonding partner of the binding site's complementary face is the Asn107 backbone CO, which accepts a hydrogen bond from the water molecule that also binds to the Leu119 NH and the drug hydrogen bond acceptor. We find that, in the α7 receptor, ACh and epibatidine are largely insensitive to the V108Vah mutation that attenuates the hydrogen bond acceptor ability of the Asn107 CO, with EC_{50} shifts < 2-fold (Table 5.2). This group may have a modest functional relevance for varenicline, as we did record a 4.3-fold loss of function for that drug. Note that both the CO on the protein backbone and the quinoxaline N on varenicline can only act as hydrogen bond acceptors, so this hydrogen bond would need to be mediated by a water molecule, as observed in AChBP. Analogous hydroxy acid mutations modulating this CO in the muscle-type and α4β4 receptors did not affect agonist EC_{50}.[22] Note that α-hydroxy acid mutations probing backbone CO hydrogen bonding can produce large EC_{50}

shifts, an example being the TrpB backbone CO, where we have seen shifts as large as 20-30-fold in other receptors.[25,26]

Taken together, it is possible that the α7 receptor either engages the agonist hydrogen bond acceptor with other groups or lacks energetically significant contacts with it. Regarding the former possibility, other candidate hydrogen bonding partners expected to lie near the agonist include the Gln117 and Asn107 side chains. Mutation of these to side chains without the potential to form hydrogen bonds has only modest functional effects of 4-fold or smaller, indicating that no critical interactions are present (Table 5.2). Without direct structural data for the α7 binding site, it is unclear exactly where these and other potential hydrogen bonding groups lie relative to the agonist.

Regarding the possibility that this hydrogen bond acceptor group does not form functionally important receptor contacts, it is worth remembering that tetramethylammonium (TMA), a much simpler structure that, of course, cannot participate in the hydrogen bonding interactions being probed here, has virtually the same potency and efficacy as ACh for the α7 receptor.[5,34] Heteromeric neuronal nAChRs and the muscle-type receptor also respond to TMA, but with substantially elevated EC_{50}s and/or reduced efficacies.[5,34] As such, it appears that the minimal requirements for agonist binding are more relaxed for α7. Indeed, the homomeric α7 receptor, which is phylogenetically more ancestral than the subunits of heteromeric receptors,[35] appears to have a less specialized binding site with broad pharmacology.[5] While most characterized agonists for the α7 receptor do posses a hydrogen bond acceptor moiety consistent with the canonical nicotinic pharmacophore, some α7-specific agonists lack this feature.[5]

Diminished importance of hydrogen bonding interactions for α7 relative to other nAChRs may underlie this specificity.

5.4.4 Interactions with complementary face side chains

With the critical positively charged "head group" of agonists buried within the aromatic box of the binding site's principal face, the remaining features of larger ligands might be expected to make significant receptor contacts to side chains on the complementary face of the binding site. In crystal structures of varenicline bound to AChBP, the side chains corresponding to Trp55 (the sole complementary face contributor to the aromatic box), Leu109, Gln117, and Leu119 all contact the ligand.[13,14] We find that the endogenous ligand ACh is minimally perturbed by alanine mutations to each of these residues (Table 5.2). The largest loss of function for ACh is observed for the L109A mutation (3.1-fold), which interestingly caused a modest gain of function (3.8-fold) for the larger varenicline ligand and a wild type EC_{50} for epibatidine. Steric compensation between the ligand and side chain could explain this observation. The Q117A mutant had only a small effect on all three agonists, with EC_{50} fold shifts of 1.8 to 2.6. 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 were previously shown to play an important functional role in the α7 receptor, with modifications to these side chains selectively affecting larger agonists.[23] Strikingly, the W55A mutation has only a small effect on

EC_{50}, but increases the relative efficacy of varenicline from 15% to 125% in the α4β2 receptor, this drug's intended target.[13] The substantial increase in EC_{50} we observe for W55A in the α7 receptor represents a dramatically different phenotype and indicates that this residue makes different contributions to receptor function for α7 *versus* α4β2. These findings suggest that avoiding agonist interactions with Trp55 could be a strategy to improve α4β2 selectivity over α7.

5.5 Conclusions

Our survey of potential agonist-receptor contacts for the α7 receptor reveals a unique pattern of interactions compared to 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.

5.6 Experimental

5.6.1 Molecular biology

cDNA for the rat α7 T6'S receptor and for human RIC-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 α7 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 tRNA THG73[36] was used for nonsense suppression at all sites except Val108 and Leu119, for which the opal suppressor TQOpS'[37,38] was used.

α-Hydroxy or amino acids were appended to the dinucleotide dCA and enzymatically ligated to the appropriate truncated 74mer suppressor tRNA as previously described.[39,40] Crude tRNA-amino acid or tRNA-hydroxy acid product was used without desalting, and the product was confirmed by MALDI-TOF MS on a 3-hydropicolinic 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.

5.6.2 Microinjection

Stage V–VI *Xenopus laevis* oocytes were harvested and injected with RNAs as described previously.[40] 5–25 ng of α7 mRNA was co-injected with ~20 ng of RIC-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 hrs before recording. In the case of low maximal currents sometimes observed in nonsense suppression experiments, presumably due to low expression, a second RNA injection was required 24 hrs after the first injection. As a negative control for suppression

experiments, unacylated full length tRNA was co-injected with mRNA in the same manner as charged tRNA. These control experiments yielded negligible responses for all sites studied.

5.6.3 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. 1 mL of each drug solution was applied for 15 s, followed by a 5-min wash step with ND96 buffer (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM HEPES) between each concentration. Acetylcholine chloride was purchased from Sigma-Aldrich (St. Louis, MO), (±)-epibatidine was purchased from Tocris (Bristol, United Kingdom), 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 (n_H) values for each condition were obtained by fitting the averaged, normalized dose-response data to the Hill equation.

5.7 References

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