Chapter 4: Binding interactions to the complementary subunit of the α4β4 receptor*

4.1 Abstract

The agonist binding site of nicotinic acetylcholine receptors (nAChRs) spans an interface between two subunits of the pentameric receptor. The principal component of this binding site is contributed by an α subunit, and it binds the cationic moiety of the nicotinic pharmacophore. The other part of the pharmacophore – a hydrogen bond acceptor – has recently been shown to bind to the complementary, non-α subunit, via the backbone NH of a conserved Leu. This interaction was predicted by studies of acetylcholine binding proteins (AChBPs) and confirmed by functional studies of the neuronal (CNS) nAChR, α4β2. The AChBP structures further suggested that the hydrogen bond to the backbone NH was mediated by a water molecule, and that a second hydrogen bonding interaction occurs between the water molecule and the backbone CO of a conserved Asn, also on the non-α subunit. Here we provide new insights into the nature of the interactions between the hydrogen bond acceptor of nicotinic agonists and the complementary subunit backbone. We have studied both the nAChR of the neuromuscular junction (muscle-type) and a neuronal subtype, $(\alpha4)_2(\beta4)_3$. In the muscle-type receptor, both acetylcholine (ACh) and nicotine show a strong interaction with the Leu NH, but the potent nicotine analog epibatidine does not. This interaction is much attenuated in the α4β4 receptor. Surprisingly, we find no evidence for a functionally significant interaction with the backbone carbonyl of the relevant Asn in either receptor with an array of agonists.

This chapter is adapted from research previously published in the Journal of Biological Chemistry:* Blum, A. P., Van Arnam, E. B., German, L. A., Lester, H. A., and Dougherty, D. A. Binding interactions to the complementary subunit of nicotinic receptors. *J. Biol. Chem.* (2013) **288, 6991–6997. *The work described in this chapter was done in collaboration with Angela P. Blum and Laurel A. German, who conducted all of the muscle-type receptor experiments.*

4.2 Introduction

nAChRs are pentamers, composed of five subunits arranged symmetrically around a central ion-conducting pore. Nicotinic agonists bind at subunit interfaces, and a combination of structure-function studies and structural studies of the acetylcholine binding proteins (AChBPs), which share considerable sequence homology with the ligand binding domain of the nAChR, have established a detailed binding model.[1-3] The α subunits contribute the principal component of the agonist binding site, which binds to the cationic end of agonists. This binding site is well-characterized, consisting of a cation-π interaction to one of several conserved aromatic residues and typically a hydrogen bond from the N^+H of the drug to a backbone carbonyl.[4-6] The natural agonist acetylcholine (ACh), which lacks the crucial N^+H, does not participate in the latter interaction.

The complementary component of the agonist binding site is formed by non-α subunits, and recent work has shown that it involves a hydrogen bonding interaction to the hydrogen bond acceptor of agonists (e.g., the C=O of ACh or the pyridine N of nicotine; Figures 4.1 and 4.2). Crystal structures of AChBPs with several drugs bound produced a binding model in which two backbone features – a CO and an NH from amino acids that are 12 residues apart – coordinate a water molecule, which in turn hydrogen bonds to the hydrogen bond acceptor of agonists (Figure 4.1).[2,7,8] In nAChRs the particular residues are an Asn and a Leu, and they are conserved across the family (Figure 4.3; they are a Leu and a Met, respectively, in the AChBP structure of Figure 4.1A). Since residue numbering varies among different receptors, we will refer to them simply as the Asn and the Leu sites, the former contributing a CO, the latter an NH to the

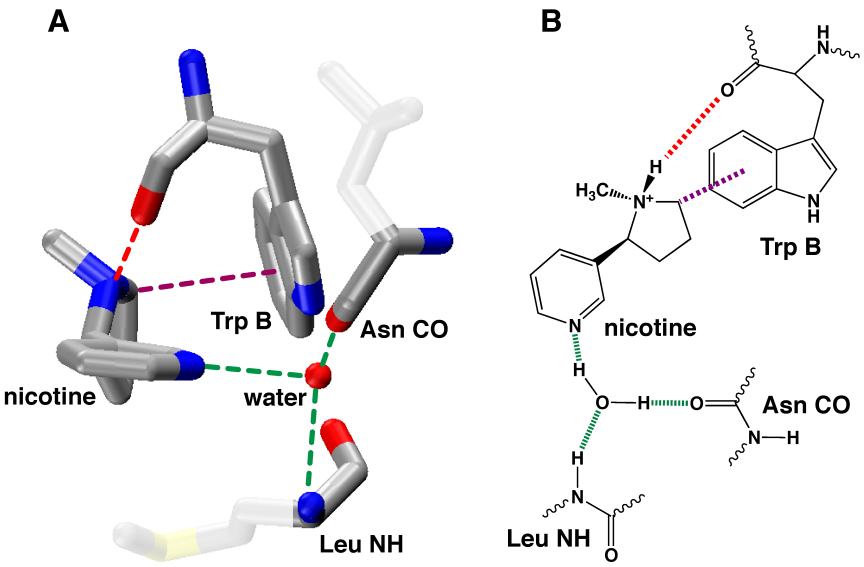

Figure 4.1. Proposed binding model for nicotine at nAChRs. **(A)** Crystal structure of nicotine bound to AChBP (1UW6). **(B)** Schematic of binding model, denoting key interactions probed here.

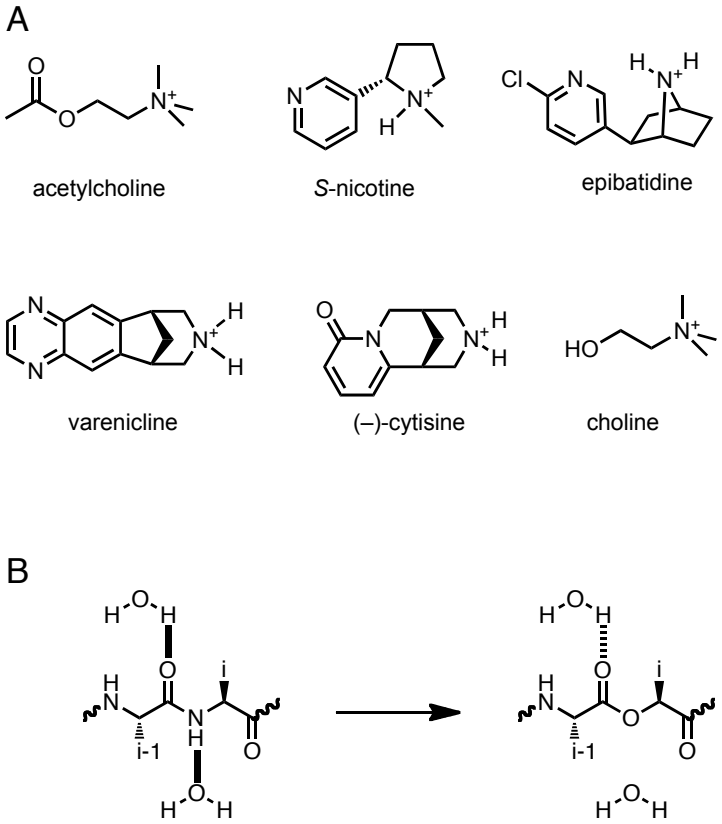

Figure 4.2. **(A)** Agonists used in this study. **(B)** Illustration of amide-to-ester mutation. Introduction of an α-hydroxy acid in place of an amino acid eliminates the hydrogen bond donor (backbone NH) of the *i* residue and attenuates the hydrogen bond accepting ability of the *i-1* carbonyl. In this figure, the attenuated hydrogen bond is represented by a dashed line.

	104																		123
rat β2	F	Y	S	**N**	A	V	V	S	Y	D	G	S	I	F	W	**L**	P	P	A	I
human β4	V	Y	T	**N**	L	I	V	R	S	N	G	S	V	L	W	**L**	P	P	A	I
mouse γ	L	Y	C	**N**	V	L	V	S	P	D	G	C	I	Y	W	**L**	P	P	A	I
mouse δ	Y	A	C	**N**	V	L	V	Y	D	S	G	Y	V	T	W	**L**	P	P	A	I
AChBP (*Lymnaea*)	T	P	Q	**L**	A	R	V	V	S	D	G	E	V	L	Y	**M**	P	S	I	R

Figure 4.3. Sequences of the complementary subunits considered here. The hydrogen bond-donating Leu and hydrogen bond-accepting Asn are highlighted. The key residues are highly conserved in other orthologs. Residue numbering is for the β2 subunit.

proposed hydrogen bonding array; specific residue numbers are noted in the experimental section. Recent studies of the neuronal α4β2 nAChR confirmed that the Leu NH of the β2 subunit does hydrogen bond to the pyridine N of nicotine and to the carbonyl O of ACh.[9]

The present work expands these studies of hydrogen bonding interactions involving the complementary subunit in two ways. First, we consider two new receptor subtypes: a second neuronal form, $(\alpha4)_2(\beta4)_3$, henceforth referred to as α4β4; and the form found at the neuromuscular junction of the peripheral nervous system, $(\alpha1)_2\beta1\gamma\delta$ (fetal form; in the adult variant the ε subunit replaces γ), which we will refer to as the muscle-type receptor. Note that the pharmacology of the muscle-type receptor is quite distinct from neuronal receptors such as α4β2 and α4β4, most importantly in the fact that nicotine is quite potent at these neuronal receptors but not at the receptors of the neuromuscular junction. This distinction allows smokers to become addicted to nicotine without adverse peripheral effects. Second, we evaluate the other component of the proposed hydrogen bonding model, the water-mediated hydrogen bond to the Asn carbonyl, in both the α4β4 and the muscle-type receptors. Efforts to probe the Asn backbone carbonyl in the previously studied α4β2 receptor were thwarted by technical

issues; the nonsense suppression methodology necessary for these studies was not selective/efficient enough for the present purposes.

Using unnatural amino acid mutagenesis we find key differences in the hydrogen bonding properties of specific drug-receptor combinations. Interestingly, we find no evidence for a functionally significant hydrogen bond to the Asn backbone carbonyl.

4.3 Results

4.3.1 General strategy

The two hydrogen bonding interactions being considered here both involve the protein backbone, and such interactions can be probed by incorporating α-hydroxy analogs of amino acids at appropriate locations (Figure 4.2B). As a probe of the Leu NH the strategy is straightforward: the backbone NH is replaced by an O. Concerning the backbone CO, α-hydroxy substitution attenuates the hydrogen bonding ability of the *i-1* carbonyl by converting it to an ester carbonyl. It is well-established that the carbonyls of esters are much poorer hydrogen bond acceptors than those of amides. Interestingly, in many studies, both quantitative and qualitative, it has been shown that the two effects associated with backbone ester incorporation – removal of the NH hydrogen bond donor and attenuation of the CO hydrogen bond acceptor – can have similar energetic consequences.[10-14] As such, to perturb the Asn CO we actually mutate the *i+1* residue, which is Leu in the α4β4 receptor and Val in the muscle-type receptor. Backbone ester mutations can be efficiently incorporated site-specifically into nAChRs expressed in *Xenopus* oocytes by nonsense suppression methodology.[15,16] Typical experimental traces

and dose-response relations for unnatural amino acid mutagenesis experiments with these receptors have been reported previously.[6,17]

These studies use EC_{50}, the effective agonist concentration needed to reach a half-maximal response, as a read-out of the functional impact of each mutation. It is well recognized that EC_{50} is a composite measure, reflecting multiple equilibria that include both "binding" events – drug entering/exiting the agonist binding site – and "gating" events – the equilibria between open and closed states of the channel. It is typical in an EC_{50} study to note an ambiguity as to whether a given mutation affects binding or gating. We would argue that in the present system, as in many similar previous studies from our lab, the ambiguity is of a different kind. Given the subtlety and precision of the modifications enabled by unnatural amino acid mutagenesis, combined with our structural knowledge of the binding site and the location of the mutations made, it is clear that we are perturbing a hydrogen bonding interaction between the drug and the receptor – a binding interaction. In order to see a change in EC_{50}, it must be true that the hydrogen bonding interaction is diminished (or enhanced) in one or more of the multiple equilibria noted above. In the studies presented here, the ambiguity in the EC_{50} measurement concerns which *equilibrium* is perturbed, not the nature of the perturbation, which is clearly an attenuated binding interaction.

Detailed kinetic analyses, typically at the single channel level, can often determine which equilibrium step(s) is being perturbed. However, we consider EC_{50} to be an 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. More importantly, our goal is to make pharmacological comparisons among closely related systems in response to subtle structural changes. We also wish to compare these results to those of previous studies on related systems. EC_{50} is a good measure of pharmacological activity. Given our experience with these systems and unnatural amino acid mutagenesis in particular, we consider EC_{50} differences of less than a factor of 2 to be not interpretable.

For studies of the muscle-type receptor we use the known L9'S mutation in the M2 transmembrane helix of the β1 subunit (where 9' is the ninth amino acid from the cytoplasmic end of the M2 α-helix).[18,19] This mutation is introduced to generically increase the sensitivity of the protein to agonists, and it results in a systematic ~40-fold decrease in EC_{50}. Given that the 9' position is ~60 Å away from the agonist binding site, this mutation is generally expected to primarily affect gating and not agonist binding, although complications can arise.[20] We have performed backbone ester mutagenesis of the Leu NH in the muscle-type receptor both in the absence and presence of the L9'S background mutation, and similar shifts in EC_{50} were seen for ACh (Table 4.1). This confirms the viability of this strategy in the present system. The agonist concentrations that were required to obtain a dose-response relation for epibatidine, nicotine and choline in the absence of the L9'S mutation were high enough that channel block by the agonist became a problem with some mutants, so all comparisons for this receptor are done using the L9'S mutation. An analogous mutation was also used in the studies of α4β2.[5] No such modification was necessary for the α4β4 receptor.

Table 4.1. Mutagenesis of the backbone NH (γL119/δL121 mutations) and backbone CO (γV108/δV110 mutations) of the muscle-type nAChR. EC_{50} and Hill coefficient (n_H) are ± SEM for goodness of fit to the Hill equation.

	Agonist	EC_{50} (μM)	Fold Shift	n_H
wild type	ACh	16.0 ± 0.3		1.3 ± 0.1
γL119Leu, δL121Leu[a]	ACh	16.0 ± 0.5		1.5 ± 0.1
γL119Lah, δL121Lah	ACh	230 ± 6	14	1.5 ± 0.1
wild type[b]	ACh	0.61 ± 0.04		1.4 ± 0.1
γL119Leu, δL121Leu[a,b]	ACh	0.31 ± 0.02		1.5 ± 0.1
γL119Lah, δL121Lah[b]	ACh	9.1 ± 0.7	29	1.6 ± 0.2
wild type[b]	Choline	840 ± 20		1.6 ± 0.1
γL119Leu, δL121Leu[a,b]	Choline	780 ± 30		1.7 ± 0.1
γL119Lah, δL121Lah[b]	Choline	1000.00 ± 0.05	1.3	1.8 ± 0.1
wild type[b]	(±)-Epibatidine	0.32 ± 0.02		1.5 ± 0.1
γL119Leu, δL121Leu[a,b]	(±)-Epibatidine	0.40 ± 0.02		1.5 ± 0.1
γL119Lah, δL121Lah[b]	(±)-Epibatidine	0.52 ± 0.03	1.3	1.6 ± 0.1
wild type[b]	*S*-Nicotine	22.0 ± 0.8		1.6 ± 0.1
γL119Leu, δL121Leu[a,b]	*S*-Nicotine	23 ± 0.7		1.7 ± 0.1
γL119Lah, δL121Lah[b]	*S*-Nicotine	230 ± 30	10	2.2 ± 0.5
γV108Val, δV110Val[a,b]	ACh	0.29 ± 0.01		1.3 ± 0.1
γV108Vah, δV110Vah[b]	ACh	0.41 ± 0.05	1.4	1.2 ± 0.2
γV108Val, δV110Val[a,b]	Choline	620 ± 20		1.4 ± 0.1
γV108Vah, δV110Vah[b]	Choline	790 ± 60	1.3	1.4 ± 0.1
γV108Val, δV110Val[a,b]	(±)-Epibatidine	0.230 ± 0.006		1.4 ± 0.1
γV108Vah, δV110Vah[b]	(±)-Epibatidine	0.240 ± 0.006	1.0	1.5 ± 0.1
γV108Val, δV110Val[a,b]	*S*-Nicotine	15 ± 1		1.2 ± 0.1
γV108Vah, δV110Vah[b]	*S*-Nicotine	33 ± 2	2.2	1.6 ± 0.1

[a] *Expression of the wild type receptor with the natural amino acid incorporated by nonsense suppression*
[b] *Receptor contains the βL9'S mutation*

Table 4.2. Mutagenesis of the backbone NH (βL119 mutations) and backbone CO (βL108 mutations) of the $(\alpha4)_2(\beta4)_3$ nAChR. EC_{50} and Hill coefficient (n_H) are ± SEM for goodness of fit to the Hill equation.

	Drug	EC_{50}	Fold Shift	Hill
β4L119Leu[a]	ACh	15.0 ± 0.7		1.42 ± 0.08
β4L119Lah	ACh	43 ± 4	2.9	1.5 ± 0.2
β4L119Leu[a]	*S*-Nicotine	2.1 ± 0.2		1.3 ± 0.1
β4L119Lah	*S*-Nicotine	5.8 ± 0.4	2.8	1.4 ± 0.1
β4L119Leu[a]	(±)-Epibatidine	0.0055 ± 0.0001		1.80 ± 0.05
β4L119Lah	(±)-Epibatidine	0.01018 ± 0.00009	1.9	1.63 ± 0.02
β4L119Leu[a]	Varenicline	0.133 ± 0.002		1.37 ± 0.02
β4L119Lah	Varenicline	0.050 ± 0.003	1 / 2.7	1.5 ± 0.1
β4L119Leu[a]	(-)-Cytisine	0.229 ± 0.004		1.37 ± 0.02
β4L119Lah	(-)-Cytisine	3.1 ± 0.1	14	1.37 ± 0.05
β4L119Leu[a]	Choline	1400 ± 300		1.4 ± 0.2
β4L119Lah	Choline	2000 ± 700	1.4	1.1 ± 0.2
β4L108Leu[a]	ACh	15.2 ± 0.9		1.43 ± 0.09
β4L108Lah	ACh	13 ± 2	1 / 1.2	1.2 ± 0.2
β4L108Leu[a]	*S*-Nicotine	1.9 ± 0.2		1.3 ± 0.1
β4L108Lah	*S*-Nicotine	1.7 ± 0.2	1 / 1.1	1.4 ± 0.1
β4L108Leu[a]	(±)-Epibatidine	0.0050 ± 0.0002		1.70 ± 0.08
β4L108Lah	(±)-Epibatidine	0.0065 ± 0.0002	1.3	1.71 ± 0.08
β4L108Leu[a]	Varenicline	0.120 ± 0.004		1.38 ± 0.05
β4L108Lah	Varenicline	0.24 ± 0.01	2.0	1.19 ± 0.05
β4L108Leu[a]	(-)-Cytisine	0.227 ± 0.005		1.42 ± 0.04
β4L108Lah	(-)-Cytisine	0.139 ± 0.007	1 / 1.6	1.40 ± 0.08
β4L108Leu[a]	Choline	1200 ± 70		1.62 ± 0.09
β4L108Lah	Choline	1100 ± 100	1 / 1.1	1.4 ± 0.1

[a]*Expression of the wild type receptor with the natural amino acid incorporated by nonsense suppression*

4.3.2 Mutagenesis studies of the Leu NH

To probe for the presumed hydrogen bond to the Leu backbone NH, the leucine (γL119/δL121 in the muscle-type receptor, βL119 in the α4β4 receptor) was replaced with its α-hydroxy acid analog (leucine, α-hydroxy; Lah). In the muscle-type receptor, ACh and nicotine both showed substantial increases in EC_{50}, (Table 4.1) confirming that the backbone NH is important for receptor activation by these agonists. Surprisingly, epibatidine, a nicotine analog that is quite potent at the muscle-type nAChR (although

~300-fold less so than at the α4β2 subtype), was unresponsive to the backbone ester mutation. This contrasts the 5-fold increase in EC_{50} seen in the α4β2 receptor for the analogous mutation with epibatidine as agonist. As expected, choline, which lacks the CO that serves as the hydrogen bond acceptor, was unresponsive to the backbone mutation, giving no shift in EC_{50} upon incorporation of the α-hydroxy acid.

Surprisingly, the analogous Leu to Lah mutation in α4β4 showed small to negligible effects for ACh, nicotine, epibatidine, varenicline, and (as expected) choline (Table 4.2). Cytisine does show a large response, establishing that the Leu NH can function as a hydrogen bond donor to an agonist in the α4β4 receptor.

4.3.3 Mutagenesis studies of the Asn CO

The second hydrogen bond predicted by the AChBP structures is to the backbone CO of a conserved Asn. To probe for a hydrogen bond to this backbone CO, the *i+1* residue, γV108/δV110 in the muscle-type receptor and βL108 in α4β4, is replaced with its α-hydroxy acid analog (valine, α-hydroxy; Vah for Val or Lah for Leu). As discussed above, this converts a backbone amide to a backbone ester, thereby attenuating the hydrogen bond-accepting ability of this moiety.

Early efforts to probe the CO of the relevant Asn residue in the α4β2 receptor gave inconsistent results that led us to question whether we could reliably control the stoichiometry of the mutant receptor.[21] Since the muscle-type receptor reliably assembles into just one stoichiometry ($(\alpha 1)_2\beta 1\gamma\delta$), we anticipated that comparable experiments would experience fewer complications, and, indeed, nonsense suppression studies at the

appropriate Val gave functional mutant receptors. However, ACh, nicotine, epibatidine, and choline were not significantly impacted by the backbone ester mutation (Table 4.1).

With the experience gained from the muscle-type receptor, we were able to probe the key Asn carbonyl in a neuronal receptor, α4β4. Again, we find no evidence for a meaningful interaction with the carbonyl for the agonists ACh, nicotine, epibatidine, varenicline, cytisine, and choline (Table 4.2).

4.4 Discussion

In recent years, the well-studied nicotinic pharmacophore, comprised of a cationic N and a hydrogen bond acceptor,[22] has been mapped onto specific binding interactions in the nAChR (Figure 4.1). The cationic N binds to the principal component of the agonist binding site in the α subunit, and the hydrogen bond acceptor binds to the complementary, non-α subunit. Guided by structures of AChBP, backbone mutagenesis studies established a hydrogen bond between the pharmacophore acceptor (pyridine N of nicotine; carbonyl O of ACh) and a Leu backbone NH in the α4β2 neuronal nAChR. It is important to note that it is not just the rise in EC_{50} resulting from backbone mutation that establishes a hydrogen bond. In all cases, choline, which lacks the hydrogen bond acceptor of the other agonists, is unaffected by the backbone mutation, proving a direct link between the mutation and the hydrogen bond acceptor of agonists. In our previous experiments with the α4β2 subtype, we also studied the nicotine analog S-N-methyl-2-phenylpyrrolidine (S-MPP).[9] In this structure, the pyridyl ring of nicotine is replaced with a phenyl ring, providing an alternative way to probe the hydrogen bond-accepting

pyridine N. This is a more subtle probe than the ACh/Ch comparison, and it provided a compelling link between the hydrogen bond acceptor of nicotine and the backbone NH in the α4β2 receptor. In the present systems, we were unable to perform comparable studies with S-MPP, because the low potency of this compound at the receptors considered here required agonist concentrations that produced competing channel block of the receptor in dose-response studies. Nevertheless, the studies of the α4β2 receptor provide support for the notion that mutations of the Leu NH are perturbing a hydrogen bond to the agonist.

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 first discuss our findings concerning the contribution of the Leu backbone NH.

ACh and nicotine both show a strong hydrogen bonding interaction with the Leu backbone NH in the muscle-type receptor. Nicotine shows very poor potency at the wild-type muscle receptor, and so we were surprised to find that nicotine is very sensitive to the Leu backbone ester mutation, more sensitive than it is to the corresponding mutation in α4β2, where nicotine is a very potent agonist. This mutation also impacted ACh potency much more in the muscle-type receptor than in the α4β2 receptor. We have performed similar backbone mutations at locations throughout the nAChR to probe for various hydrogen bonds, and we typically see informative, but modest increases in EC_{50} of ~5-20-fold. The 29-fold increase in EC50 seen for ACh in the muscle-type receptor is among the largest responses we have seen for a backbone ester mutation. It is also much

larger than the 7-fold increase that was seen for the equivalent mutation in the α4β2 receptor.[9] These results may suggest that the hydrogen bond to the Leu NH is stronger in the muscle-type receptor, and it is possible that ACh and nicotine sit more closely to this residue in the muscle-type receptor than they do in the α4β2 subtype. As expected, choline as an agonist is unresponsive to this mutation.

Surprisingly, epibatidine, a potent agonist at the muscle-type receptor, is unresponsive to the Leu backbone NH mutation. In crystal structures of AChBP binding nicotine or epibatidine, the relative positioning of all relevant atoms – the pyridine N and the backbone NH and CO – are essentially identical. As such, it has been assumed that these two closely related molecules bind in the same way, even though the bridging water is not observable in the epibatidine structure, presumably because is it not ordered enough for the relatively low resolution structure.

One possible explanation for the lack of a functionally significant hydrogen bonding interaction with the pyridine N of epibatidine in the muscle-type receptor is that the N is a relatively poor hydrogen bond acceptor. When considering closely related systems, pKa is an excellent predictor of hydrogen bonding ability. Pyridine, a good model for nicotine, has a pKa of 5.2, but 2-chloropyridine, a model for epibatidine, has a much lower pKa of 0.5.[23] Thus, epibatidine is expected to be a poorer base than nicotine by ~5 orders of magnitude, and it is safe to conclude that epibatidine would also be a much poorer hydrogen bond acceptor. Recently we have showed that varenicline, the smoking cessation compound marketed as Chantix®, is similarly unresponsive to the analogous backbone NH mutation in the α4β2 receptor.[24] The quinoxaline nitrogens of varenicline have a pKa of 0.8, quite similar to that for epibatidine. Thus, in these two

very potent nicotinic agonists – epibatidine and varenicline – the strength of the hydrogen bond acceptor is expected to be greatly attenuated, and our functional assay for this hydrogen bond appears to reflect this property.

When we probe the Leu backbone NH interaction in the α4β4 receptor, we find a much smaller impact of the backbone mutation. ACh and nicotine show meaningful, but much smaller effects than is usual; in α4β2 receptors the effect ranged from 5.6- to 8.5-fold for the same agonists. Again, epibatidine shows no meaningful effect and varenicline actually shows a small gain of function. Cytisine, another compound that is marketed for smoking cessation under the brand name Tabex®, shows a large effect. The hydrogen bond acceptor in cytisine is not a pyridine-type N, but is rather the O of an amide carbonyl. Amides are much stronger hydrogen bond acceptors, and, indeed, we saw very large effects for cytisine at the α4β2 receptor. For the two stoichiometries of α4β2 – 2:3 and 3:2 – the backbone NH mutation led to perturbations of 62- and 14-fold, respectively, with cytisine as the agonist.

The second component of the interaction with the hydrogen bond acceptor of nicotinic agonists predicted by AChBP structures is the water-mediated hydrogen bond to the Asn backbone CO. Using the backbone ester strategy to perturb this proposed interaction, we measured 9 different drug-receptor interactions involving two different receptors, and in no case do we see a meaningful interaction (not including choline, for which no effect is expected). The strongest effects are for varenicline at α4β4, with a ratio of 2.0, and for nicotine at the muscle-type receptor, with a ratio of 2.2, barely what we consider to be meaningful. All other effects are less than a factor of two.

Note that the strategy employed here to probe a hydrogen bond to a backbone carbonyl can produce large effects. In nicotinic receptors when we use the strategy to probe the interaction of the N^+H of the drug to the backbone carbonyl of the key Trp residue of the binding site (Figure 4.1), we see EC50 shifts ranging from 9- to 27-fold for potent drug-receptor combinations.

We thus conclude that the water-mediated interaction between the hydrogen bond acceptor of agonists and the Asn backbone CO seen in AChBP structures is not functionally significant in nAChRs in general. We note that there is a fundamental distinction between the two hydrogen bonds seen in AChBP. If the water molecule were not present, the Leu backbone NH could donate a hydrogen bond directly to the hydrogen bond acceptor of agonists. In contrast, the Asn backbone CO is itself a hydrogen bond acceptor, and so it can interact with the hydrogen bond acceptor of agonists only through an intermediary water. Our results thus open up the possibility that the water molecule that is seen in essentially all AChBP structures is not present in actual receptors. Whether the water molecule is or is not present, we find that perturbing its putative hydrogen bonding partner has little consequence on receptor function.

In the pharmacology of nicotinic receptors, it has often been suggested that the non-α, complementary subunit plays a key role in establishing subtype selectivity for various drugs. We have now probed the complementary binding site for four nicotinic subtypes (muscle-type, α4β4, and both stoichiometries of α4β2), and see interesting variations for particular drug-receptor combinations. We believe this information will be of value to efforts to develop more selective drugs that target nicotinic receptors.

We have shown that ACh and nicotine both engage in a functionally important hydrogen bond to the complementary subunit Leu backbone NH in the muscle-type nAChR, but the nicotine analog epibatidine does not. In the α4β4 receptor, interactions with the Leu backbone NH are surprisingly weak. We also find no evidence for a functionally important water-mediated hydrogen bond to the Asn backbone CO. Our results highlight the necessity of functional studies on intact receptors to probe interactions suggested by structural studies of model systems.

4.5 Experimental

4.5.1 Mutagenesis

Nonsense suppression was performed using techniques described previously on the mouse muscle embryonic nAChR, $(\alpha 1)_2\beta 1\gamma\delta$, in the pAMV vector[5] and human $(\alpha 4)_2(\beta 4)_3$ receptor in the pGEMhe vector. For nonsense suppression experiments, a TAG (for mutation at γV108/δV110) or TGA stop codon (for mutation at γL119/δL121, β4L108, and β4L119) was introduced at the site of interest by the standard Stratagene QuickChange protocol and verified through sequencing. The β1 subunit contains a background mutation in the transmembrane M2 helix (β1L9'S) that is known to lower whole-cell EC_{50} values. The α1 subunit contains a hemagglutinin epitope in the M3-M4 cytoplasmic loop that does not alter EC_{50} values in control experiments. cDNA was linearized with the restriction enzyme NotI for muscle-type receptor subunits and NheI for α4 and β4. mRNA was prepared by *in vitro* transcription using the mMessage Machine T7 kit (Ambion).

4.5.2 Microinjection

Stage V-VI *Xenopus laevis* oocytes were injected with mRNA in a 10:1:1:1 or 1:1:5:5 ratio of α1:β1:γ:δ for wild-type/conventional mutagenesis or nonsense suppression experiments, respectively, on the muscle-type receptor. An mRNA ratio of 1:20 (α4:β4) was used for nonsense suppression experiments on the $(\alpha4)_2(\beta4)_3$ receptor. Control nonsense suppression experiments confirmed that this ratio ensures a 2:3 subunit stoichiometry. α-Hydroxy acids and amino acids were appended to the dinucleotide dCA and enzymatically ligated to the truncated 74-nucleotide amber suppressor tRNA THG73 or opal suppressor tRNA TQOpS' as previously described.[5] For wild-type or conventional mutagenesis experiments on the muscle-type receptor, 1-2 ng of mRNA was injected per oocyte in a single 75 nL injection. For nonsense suppression experiments on the muscle-type receptor, each cell was injected with 75 nL of a 1:1 mixture of mRNA (20-25 ng of total mRNA) and tRNA (10-25 ng). For nonsense suppression experiments on the $(\alpha4)_2(\beta4)_3$ receptor, each cell was injected with 50 nL of a 1:1 mixture of mRNA (50 ng total) and tRNA (~25 ng). 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. Oocytes were incubated at 18 °C for 16-20 or 24-48 hours after injection for the wild-type/conventional mutagenesis or nonsense suppression experiments, respectively, on the muscle-type receptor. Oocytes were incubated for 48 hours after injection for nonsense suppression experiments on the $(\alpha4)_2(\beta4)_3$ receptor. Wild-type recovery control experiments (injection of tRNA appended to the natural amino acid) were performed to evaluate the fidelity of the nonsense suppression experiments. In additional control experiments on

the muscle-type receptor, injections of mRNA only and mRNA with 76-mer THG73 or TQOpS' gave minimal currents in electrophysiology experiments (~100 nA or less for controls compared to >>2 μA for nonsense suppression experiments). For the $(\alpha4)_2(\beta4)_3$ receptor, injections of mRNA with 76-mer TQOpS' gave no detectable currents.

4.5.3 Electrophysiology

Two-electrode voltage clamp electrophysiology was used to measure the functional effects of each mutation. Electrophysiology recordings were performed after injection and incubation as described above using the OpusXpress 6000A (Axon Instruments) at a holding potential of −60 mV. The running buffer was a Ca^{2+} free ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.5). Agonist doses in Ca^{2+}-free ND96 were applied for 15 s followed by a 116 s wash with the running buffer. Acetylcholine chloride, (−)-nicotine tartrate, and (−)-cytisine were purchased from Sigma-Aldrich/RBI, (±)-epibatidine was purchased from Tocris, and varenicline tartrate was a generous gift from Pfizer. Dose-response data were obtained for ≥ 8 agonist concentrations on ≥ 8 cells. Dose-response relations were fit to the Hill equation to obtain EC_{50} and Hill coefficient values, which are reported as averages ± standard error of the fit.

4.6 References

1. Brejc, K. *et al.* Crystal structure of an ACh-binding protein reveals the ligand-binding domain of nicotinic receptors. *Nature* **411,** 269–276 (2001).
2. Celie, P. H. *et al.* Nicotine and carbamylcholine binding to nicotinic acetylcholine receptors as studied in AChBP crystal structures. *Neuron* **41,** 907–914 (2004).
3. Sixma, T. K. & Smit, A. B. Acetylcholine binding protein (AChBP): a secreted glial protein that provides a high-resolution model for the extracellular domain of

pentameric ligand-gated ion channels. *Annu. Rev. Biophys. Biomol. Struct.* **32,** 311–334 (2003).

4. Cashin, A. L., Petersson, E. J., Lester, H. A. & Dougherty, D. A. Using physical chemistry to differentiate nicotinic from cholinergic agonists at the nicotinic acetylcholine receptor. *J. Am. Chem. Soc.* **127,** 350–356 (2005).
5. Xiu, X., Puskar, N. L., Shanata, J. A., Lester, H. A. & Dougherty, D. A. Nicotine binding to brain receptors requires a strong cation-pi interaction. *Nature* **458,** 534–537 (2009).
6. Zhong, W. *et al.* From ab initio quantum mechanics to molecular neurobiology: a cation-pi binding site in the nicotinic receptor. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 12088–12093 (1998).
7. Hansen, S. B. *et al.* Structures of Aplysia AChBP complexes with nicotinic agonists and antagonists reveal distinctive binding interfaces and conformations. *EMBO J* **24,** 3635–3646 (2005).
8. Hansen, S. B. *et al.* Structural characterization of agonist and antagonist-bound acetylcholine-binding protein from Aplysia californica. *J. Mol. Neurosci.* **30,** 101–102 (2006).
9. Blum, A. P., Lester, H. A. & Dougherty, D. A. Nicotinic pharmacophore: the pyridine N of nicotine and carbonyl of acetylcholine hydrogen bond across a subunit interface to a backbone NH. *Proc. Natl. Acad. Sci. U.S.A.* **107,** 13206–13211 (2010).
10. Deechongkit, S. *et al.* Context-dependent contributions of backbone hydrogen bonding to beta-sheet folding energetics. *Nature* **430,** 101–105 (2004).
11. Deechongkit, S., Dawson, P. E. & Kelly, J. W. Toward assessing the position-dependent contributions of backbone hydrogen bonding to beta-sheet folding thermodynamics employing amide-to-ester perturbations. *J. Am. Chem. Soc.* **126,** 16762–16771 (2004).
12. England, P. M., Zhang, Y., Dougherty, D. A. & Lester, H. A. Backbone mutations in transmembrane domains of a ligand-gated ion channel: implications for the mechanism of gating. *Cell* **96,** 89–98 (1999).
13. Gleitsman, K. R., Kedrowski, S. M., Lester, H. A. & Dougherty, D. A. An intersubunit hydrogen bond in the nicotinic acetylcholine receptor that contributes to channel gating. *J. Biol. Chem.* **283,** 35638–35643 (2008).
14. Koh, J. T., Cornish, V. W. & Schultz, P. G. An experimental approach to evaluating the role of backbone interactions in proteins using unnatural amino acid mutagenesis. *Biochemistry* **36,** 11314–11322 (1997).
15. Nowak, M. W. *et al.* In vivo incorporation of unnatural amino acids into ion channels in Xenopus oocyte expression system. *Meth. Enzymol.* **293,** 504–529 (1998).
16. Nowak, M. W. *et al.* Nicotinic receptor binding site probed with unnatural amino acid incorporation in intact cells. *Science* **268,** 439–442 (1995).
17. Puskar, N. L., Xiu, X., Lester, H. A. & Dougherty, D. A. Two neuronal nicotinic acetylcholine receptors, alpha4beta4 and alpha7, show differential agonist binding modes. *J. Biol. Chem.* **286,** 14618–14627 (2011).
18. Labarca, C. *et al.* Channel gating governed symmetrically by conserved leucine residues in the M2 domain of nicotinic receptors. *Nature* **376,** 514–516 (1995).

19. Filatov, G. N. & White, M. M. The role of conserved leucines in the M2 domain of the acetylcholine receptor in channel gating. *Mol Pharmacol* **48,** 379–384 (1995).
20. Gleitsman, K. R., Shanata, J. A., Frazier, S. J., Lester, H. A. & Dougherty, D. A. Long-range coupling in an allosteric receptor revealed by mutant cycle analysis. *Biophys J* **96,** 3168–3178 (2009).
21. Blum, A. P. Structure-Function Studies of Nicotinic Acetylcholine Receptors Using Unnatural Amino Acid Analogs and Synthetic Agonist Analogs. (2011).
22. Beers, W. H. & Reich, E. Structure and activity of acetylcholine. *Nature* **228,** 917–922 (1970).
23. Linnell, R. Dissociation Constants of 2-Substituted Pyridines. *J Org Chem* **25,** 290 (1960).
24. Da Silva Tavares, X. *et al.* Variations in binding among several agonists at two stoichiometries of the neuronal, alpha4beta2 nicotinic receptor. *J. Am. Chem. Soc.* **134,** 11474–11480 (2012).