CHAPTER 3: Residues that Contribute to Binding of the Nicotinic Pharmacophore in the Muscle-Type Nicotinic Receptor*

3.1 ABSTRACT

The agonist binding site of nicotinic acetylcholine receptors (nAChRs) spans an interface between two subunits of the pentameric receptor. The principal component 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. Studies of the neuronal (CNS) receptor α4β2 show that the backbone NH of Leu119 is the donor to the acceptor on the agonist, an interaction presaged by studies of the structurally homologous acetylcholine binding proteins (AChBP). The AChBP structures further suggested that the hydrogen bond to Leu119 was mediated by a water molecule, and that a second hydrogen bonding interaction occurs to the backbone CO of Asn107, also on the complementary subunit. Here we provide new insights into the nature of the interactions between the hydrogen bond acceptor of nicotinic agonists and the backbone features of the complementary subunit. We find that, like the neuronal receptor, the nAChR of the neuromuscular junction (muscle-type) shows a strong interaction with Leu119 (γL119/δL121 in muscle-type receptor numbering) for both ACh and nicotine. However, we find no evidence for a functionally significant interaction with Asn107 (γN107/δN109). Surprisingly, the potent nicotine analog epibatidine does not make a functionally important hydrogen bond to

* *This work was done in collaboration with Laurel A. German, a high school student from Polytechnic School.*

either γL119/δL121 nor γN107/δN109. In addition, a mutation that has been shown to profoundly affect interactions to the principal component of the agonist binding site of the muscle-type receptor, Gly153Lys, has no impact on interactions involving the complementary subunit.

3.2 INTRODUCTION

Nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels that propagate neurotransmission in the central and peripheral nervous systems and are activated by the neurotransmitter acetylcholine and also by nicotine.[1-3] The nAChRs are members of a superfamily of ligand-gated ion channels called the Cys-loop (or pentameric) receptors, which also includes receptors for the neurotransmitters γ-aminobutyric acid ($GABA_A$ and $GABA_C$), glycine (GlyR) and serotonin ($5\text{-}HT_3$). The family is implicated in an assortment of neurological disorders including Alzheimer's disease, Parkinson's disease, schizophrenia, and depression, and are also essential for learning, memory and sensory perception.[4, 5]

nAChRs are pentamers, composed of five subunits arranged symmetrically around a central ion-conducting pore.[1-3] There are 16 mammalian genes that encode 16 homologous but functionally distinct nAChR subunits (α1-α7, α9, α10, β1-β4, γ, δ, ε). From various combinations of these subunits, >20 active nAChR subtypes have been established. Of the various nAChR subtypes, the heteropentameric $\alpha1_2\beta1\gamma\delta$ is the most studied, owing to its precise subunit stoichiometry and abundance in the electric organ of eels and rays, which facilitated many early studies of nAChRs.[1] In humans, this subtype is expressed post-synaptically at neuromuscular junctions of the peripheral nervous system and is therefore referred to as the 'muscle-type' receptor. Other nAChRs mediate

synaptic transmission between nerve cells in the central nervous system and autonomic ganglia and are collectively referred to as "neuronal" subtypes. These include α4β2, which is strongly implicated in nicotine addiction[6-9] and is the target of the recently developed smoking cessation drug Chantix® (varenicline).[6]

The nicotinic pharmacophore is one of the longest-known, best-studied pharmacophores, and it is comprised of a cationic nitrogen and a hydrogen bond acceptor.[10, 11] Agonists bind at subunit interfaces,[1-3] and a combination of structure-function studies[12-16] and structural studies of the acetylcholine binding proteins (AChBP),[17-22] which share considerable sequence homology with the ligand binding domain of the nAChR, have mapped binding interactions of the pharmacophore onto these interfaces. 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 (**Figure 3.1**), consisting of a cation-π interaction to one of several conserved aromatic residues and a hydrogen bond from the N^+H of the drug to a backbone carbonyl (except in the case of ACh, which cannot serve as a hydrogen bond donor).[13-16]

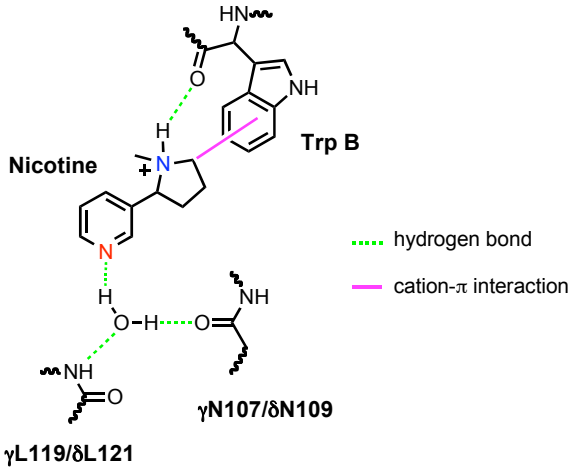

Figure 3.1. Depiction of binding interactions of the nicotinic pharmacophore as predicted by AChBP structures.[18, 20] Residue numbering is for the muscle-type receptor. TrpB is from the principal subunit (α1).

The complementary component of the agonist binding site is formed by non-α subunits, and recent work has shown that it makes a hydrogen bonding interaction to the hydrogen bond acceptor of agonists. Crystal structures of AChBPs with several drugs bound produced a model in which two backbone residues – the NH of Leu119 and the CO of Asn107 – coordinate a water molecule, which in turn hydrogen bonds to the hydrogen bond acceptor of agonists (**Figure 1.3**).[18, 20] Recent studies of the neuronal α4β2 receptor confirmed that the NH of Leu119 of the β2 subunit does hydrogen bond to the pyridine N of nicotine and to the carbonyl O of ACh.[12]

Note that the pharmacology of the muscle-type receptor is quite distinct from neuronal receptors such as α4β2, most importantly in the fact that nicotine is quite potent at the neuronal receptor[15] but not at the receptors of the neuromuscular junction.[13] This distinction allows smokers to become addicted to nicotine without adverse peripheral effects.[15] We have shown that binding interactions in the principal component of the agonist binding site strongly differentiate the interaction of nicotine with the two receptor subtypes. ACh, nicotine[15] and epibatidine (See Chapter 4) each form strong cation-π interactions in α4β2, but only ACh and epibatidine make the cation-π interaction in the muscle-type receptor.[13] Similarly, the hydrogen bond to the backbone CO of the principal subunit is less sensitive to mutation in the muscle-type receptor than in the α4β2 subtype.[13, 15]

The present work focuses on the complementary component of the agonist binding site of the muscle-type nAChR. We wished to know whether the hydrogen bond to the NH of Leu119 was important for agonist binding in the muscle-type receptor, as it is in α4β2. We also sought to determine a role for the backbone CO of Asn107. An

important residue in distinguishing the pharmacology of the muscle-type from α4β2 is at site 153 of the α subunit, where the α1G153K mutation in the muscle-type substantially alters interactions in the principal component of the agonist binding site, thereby greatly increasing nicotine affinity.[15, 23] We wished to determine whether this mutation also impacts the agonist binding interactions to the complementary component of the agonist binding site.

Using unnatural amino acid mutagenesis and mutant cycle analysis we find that the hydrogen bond to Leu119 (γL119/δL121 in muscle-type receptor numbering) is active in the muscle-type receptor as seen previously in the neuronal α4β2 subtype. However, we find no interaction with the backbone CO of N107 (γN107/δN109). Interestingly, the close nicotine homologue epibatidine shows no hydrogen bonding interaction to either residue in the complementary subunit. Finally, we find that the α1G153K mutation that profoundly affects binding interactions in the principal subunit has no effect on binding interactions involving the complementary subunit.

3.3 RESULTS

3.3.1 General Strategy

Potential hydrogen bonds to a protein backbone can be probed by replacing the residue that contributes the backbone NH with its α-hydroxy acid analog.[24-27] This mutation eliminates a hydrogen bond donor by replacing the backbone NH with an O (**Figure 3.2B**). In addition, the 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 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.[12, 24-31] Thus, incorporation of an α-hydroxy acid can probe the hydrogen bonding ability of the associated amide NH and amide carbonyl and, if either are important for agonist binding, the appropriate backbone ester mutation should have an impact on agonist potency. Backbone ester mutations can be efficiently incorporated site-specifically into nAChRs expressed in *Xenopus* oocytes by nonsense suppression methodology.[12, 28, 30, 31]

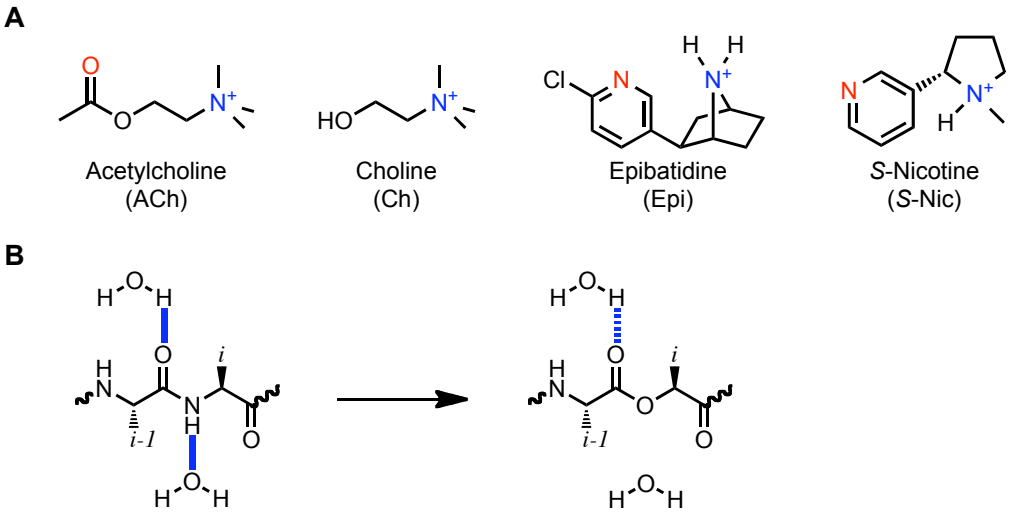

Figure 3.2. Agonists and unnatural amino acids used in this study. (A) Agonists used in this study with pharmacophore highlighted: the positively charged nitrogen is shown in blue and the hydrogen bond acceptor is shown in red. (B) Illustration of amide-to-ester mutation. Introduction of an α-hydroxy 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.

These studies use EC_{50}, the effective agonist concentration needed to reach a half-maximal response, as a readout of the functional impact of each mutation. EC_{50} is a composite measure that is influenced by agonist binding and also by channel gating (pore

opening). We do not distinguish these mechanisms here, but we feel that for studies of comparative pharmacology, EC_{50} is an acceptable metric.

In the α4β2 receptor, removal of the backbone NH of the residue analogous to γL119/δL121 had a measurable impact on receptor function, but that alone does not establish a hydrogen bond to the agonist. As such, we used double mutant cycle analysis to verify the interaction between ACh and the backbone NH of the analogous γL119/δL121 residue.[12] Mutant cycle analysis is the standard method to determine whether two mutations are energetically coupled.[32] EC_{50}-based mutant cycle analysis has been used to probe interactions in Cys-loop receptors by many labs, including our own.[12, 28, 30, 31, 33-35] It is also standard practice to convert the coupling coefficient (Ω) obtained from a mutant cycle analysis into a free energy by the equation $\Delta\Delta G° = -RT\ln(\Omega)$, where $\Omega = [EC_{50}(WT) * EC_{50}(\text{double mutant})] / [EC_{50}(\text{mutant 1}) * EC_{50}(\text{mutant 2})]$.[32] In these experiments, we are using the backbone ester mutation as the first mutation and choline (an analog of ACh that lacks the hydrogen bond accepting acetyl) as the second, thus removing or attenuating both the hydrogen bond donor and acceptor of the presumed interaction. A coupling energy of greater than 1 kcal/mol is generally regarded as compelling evidence in support of the interaction being probed.

In our previous experiments with the α4β2 subtype, we also performed a mutant cycle analysis with the nicotine analog *S*-*N*-methyl-2-phenylpyrrolidine (*S*-MPP).[12] In this structure, the pyridyl ring of nicotine is replaced with a phenyl ring, thus removing the hydrogen bond-accepting pyridine N. This is certainly a much more subtle probe than the ACh/Ch comparison. However, given the low potency of nicotine at the muscle-type

receptor, it is not surprising that *S*-MPP is a very poor agonist that cannot be studied due to channel block at the concentrations needed to observe dose-response relationships.

These studies use the known L9'S mutation in the M2 transmembrane region of the β1 subunit (where 9' is ninth amino acid from the cytoplasmic end of the M2 transmembrane α-helix).[36, 37] 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 expected to primarily effect gating and not agonist binding. The backbone ester mutation was performed at γL119/δL121 in the absence and presence of the L9'S background mutation and gave similar shifts in EC_{50} for ACh (**Table 3.1**), suggesting that the L9'S mutation does not have a substantial influence on agonist binding to this residue. 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 in the range of channel block, so all comparisons are done using this mutation. An analogous mutation was also used in the studies with α4β2.[12]

Table 3.1. EC_{50} and Hill coefficient (± standard error of the mean) values for mutations made to $\alpha 1_2\beta 1\gamma\delta$. The fold-shift is the ratio of the nonsense suppression EC_{50} values of the ester mutant over the natural amino acid. Fold-shifts previously reported for experiments[12] with α4β2 are given in parentheses. Mutations identified as "Leu" and "Val" represent recovery of the wild-type receptor by nonsense suppression.

Agonist	Mutation	EC_{50} nM	Fold-Shift	Hill
ACh	α1β1γδ	16000 ± 300		1.3 ± 0.1
	α1β1γ(L119Leu)δ(L121Leu)	16000 ± 500		1.5 ± 0.1
	α1β1γ(L119Lah)δ(L121Lah)	230000 ± 6000	14	1.5 ± 0.1
ACh	α1β1(L9'S)γδ	610 ± 40		1.4 ± 0.1
	α1β1(L9'S)γ(L119Leu)δ(L121Leu)	310 ± 20		1.5 ± 0.1
	α1β1(L9'S)γ(L119Lah)δ(L121Lah)	9100 ± 700	29 (7)	1.6 ± 0.2
Ch	α1β1(L9'S)γδ	840000 ± 20000		1.6 ± 0.1
	α1β1(L9'S)γ(L119Leu)δ(L121Leu)	780000 ± 30000		1.7 ± 0.1
	α1β1(L9'S)γ(L119Lah)δ(L121Lah)	1000000 ± 50	1.3 (1)	1.8 ± 0.1
±-Epi	α1β1(L9'S)γδ	320 ± 20		1.5 ± 0.1
	α1β1(L9'S)γ(L119Leu)δ(L121Leu)	400 ± 20		1.5 ± 0.1
	α1β1(L9'S)γ(L119Lah)δ(L121Lah)	520 ± 30	1.3 (5)	1.6 ± 0.1
S-Nic	α1β1(L9'S)γδ	22000 ± 800		1.6 ± 0.1
	α1β1(L9'S)γ(L119Leu)δ(L121Leu)	23000 ± 700		1.7 ± 0.1
	α1β1(L9'S)γ(L119Lah)δ(L121Lah)	230000 ± 30000	10 (7)	2.2 ± 0.5
ACh	α1β1(L9'S)γ(V108Val)δ(V110Val)	290 ± 10		1.3 ± 0.1
	α1β1(L9'S)γ(V108Vah)δ(V110Vah)	410 ± 50	1.4	1.2 ± 0.2
Ch	α1β1(L9'S)γ(V108Val)δ(V110Val)	620000 ± 20000		1.4 ± 0.1
	α1β1(L9'S)γ(V108Vah)δ(V110Vah)	790000 ± 60000	1.3	1.4 ± 0.1
±-Epi	α1β1(L9'S)γ(V108Val)δ(V110Val)	230 ± 6		1.4 ± 0.1
	α1β1(L9'S)γ(V108Vah)δ(V110Vah)	240 ± 6	1.0	1.5 ± 0.1
S-Nic	α1β1(L9'S)γ(V108Val)δ(V110Val)	15000 ± 1000		1.2 ± 0.1
	α1β1(L9'S)γ(V108Vah)δ(V110Vah)	33000 ± 2000	2.2	1.6 ± 0.1
ACh	α1(G153K)β1(L9'S)γδ	7.2 ± 0.7		1.3 ± 0.1
	α1(G153K)β1(L9'S)γ(L119Leu)δ(L121Leu)	7.6 ± 0.7		1.8 ± 0.3
	α1(G153K)β1(L9'S)γ(L119Lah)δ(L121Lah)	180 ± 20	24	1.3 ± 0.1
Ch	α1(G153K)β1(L9'S)γδ	30000 ± 2000		1.0 ± 0.1
	α1(G153K)β1(L9'S)γ(L119Leu)δ(L121Leu)	27000 ± 2000		1.1 ± 0.1
	α1(G153K)β1(L9'S)γ(L119Lah)δ(L121Lah)	68000 ± 3000	3	1.3 ± 0.1
±-Epi	α1(G153K)β1(L9'S)γδ	4.3 ± 0.5		0.77 ± 0.5
	α1(G153K)β1(L9'S)γ(L119Leu)δ(L121Leu)	2.3 ± 0.4		0.74 ± 0.1
	α1(G153K)β1(L9'S)γ(L119Lah)δ(L121Lah)	9.6 ± 0.4	4	1.1 ± 0.1
S-Nic	α1(G153K)β1(L9'S)γδ	320 ± 30		1.4 ± 0.2
	α1(G153K)β1(L9'S)γ(L119Leu)δ(L121Leu)	360 ± 40		0.95 ± 0.1
	α1(G153K)β1(L9'S)γ(L119Lah)δ(L121Lah)	6500 ± 500	18	1.3 ± 0.1

3.3.2 Mutagenesis studies of γL119/δL121

To probe for the presumed hydrogen bond to the backbone NH of γL119/δL121 in the muscle-type nAChR, the leucine was replaced with its α-hydroxy acid analog (leucine, α-hydroxy; Lah). ACh and nicotine showed substantial increases in EC_{50}, confirming that the backbone NH is important for receptor activation by these agonists (**Table 3.1**). We have performed similar backbone mutations at locations throughout the nAChRs to probe for various hydrogen bonds and typically see informative, but modest increases in EC_{50}

of ~5−20-fold.[12, 28, 31] The 29-fold increase in EC_{50} seen for ACh 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. The responsiveness of nicotine to the backbone ester mutation was also interesting, given its lack of participation in the cation-π interaction in the muscle-type receptor.[13] The agonist epibatidine, however, was unresponsive to backbone ester mutation, in contrast to the 5-fold increase in EC_{50} seen in the α4β2 receptor for the analogous mutation.

As expected, choline was unresponsive to the backbone mutation, giving no shift in EC_{50} upon incorporation of the α-hydroxy acid. Mutant cycle analysis between γL119Lah/δL121Lah and ACh/choline gave a large energetic coupling of 1.9 kcal/mol (**Table 3.2** and **Figure 3.3**), which is nearly double the value seen for analogous mutations in the α4β2 receptor.

Table 3.2. Comparison of coupling coefficients (Ω) and coupling energies (ΔΔG°) for double mutant cycles. Corresponding values obtained from experiments with α4β2[12] are given in parentheses.

Agonist	Mutant	Ω	ΔΔG° (kcal/mol)
ACh/Ch	α1β1(L9'S)γ(L119Lah)δ(L121Lah)	0.044 (0.16)	1.9 (1.1)
ACh/Ch	α1β1(L9'S)γ(V108Vah)δ(V110Vah)	0.90	0.061
ACh/Ch	α1(G153K)β1(L9'S)γ(L119Lah)δ(L121Lah)	0.11	1.3

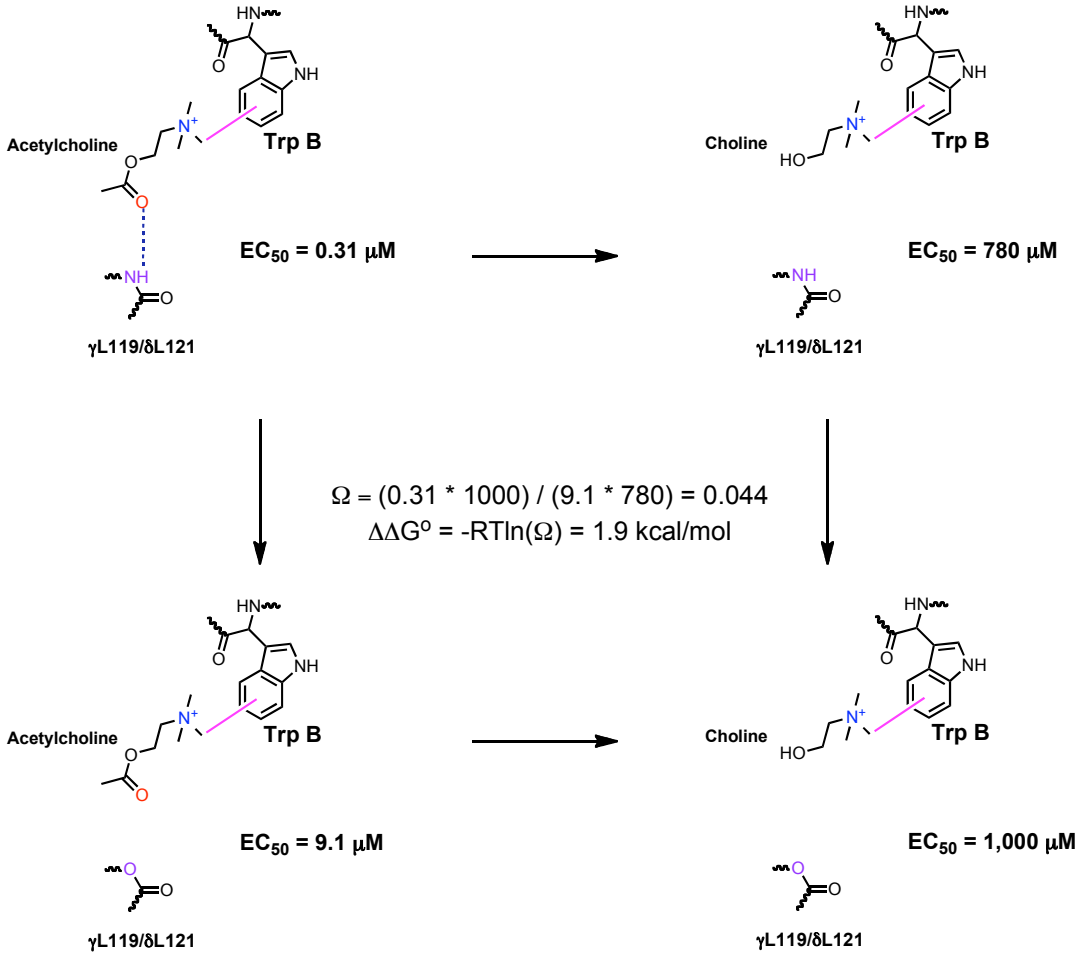

Figure 3.3. Double mutant cycle analysis for ACh and choline on wild-type and α1β1(L9'S)γ(L119Lah)/δ(L121Lah) mutant receptors.

3.3.3 Mutagenesis studies of the backbone CO of γN107/δN109

The second hydrogen bond predicted by the AChBP structures is to the backbone CO of γN107/δN109. Like γL119/δL121, this residue is thought to coordinate the water molecule that hydrogen bonds to the pyridine nitrogen of nicotine. To probe for a hydrogen bond to this backbone CO, the *i+1* residue, γV108/δV110, is replaced with its α-hydroxy acid analog (valine, α-hydroxy; Vah). As discussed above, this converts a backbone amide to a backbone ester and in doing so places an electron-withdrawing O

next to the backbone CO in question, thereby attenuating the hydrogen bond accepting ability of this moiety (**Figure 3.2B)**.

Early efforts to probe the CO of the residue analogous to γN107/δN109 in the α4β2 receptor gave inconsistent results that led us to question whether we could reliably control the stoichiometry of the mutant receptor (See Chapter 2). Since the muscle-type receptor has just one possible stoichiometry ($\alpha 1_2\beta 1\gamma\delta$), we anticipated that comparable experiments would experience fewer complications, and, indeed, nonsense suppression studies at γV108/δV110 gave functional mutant receptors. However, ACh, nicotine, epibatidine and choline were all unresponsive to the backbone ester mutation (**Table 3.1**). A mutant cycle analysis between γV108Vah/δV110Vah and ACh/choline gives simple additivity (Ω = 0.90), indicating no energetic coupling (ΔΔG° = 0.061 kcal/mol) (**Table 3.2**). These data are inconsistent with the second hydrogen bond predicted by the AChBP structures and strongly suggest that γN107/δN109 does not play a significant functional role in the nAChRs.

3.3.4 Impact of the α1G153K mutation

We have shown previously that introduction of a single mutation in the α1 subunit of the muscle-type receptor, α1G153K, has dramatic effects on the EC_{50} for nicotine, and that the increased potency of nicotine is, at least in part, a consequence of an enhanced cation-π interaction to nicotine in the mutant muscle-type receptor.[15] The α1G153 residue is located just four residues from the cation-π binding residue, TrpB, and is a Lys in the high affinity α4β2 subtype, but a Gly in the muscle-type receptor and also in other low affinity subtypes like the α7 homopentamer. It is proposed that when a Lys (or any residue other than Gly) is present at this position, a backbone hydrogen bond is formed

between the protein segment containing α1G153 "loop B" and another protein segment "loop C," which, in turn, shapes the agonist binding site in such a way that favors formation of the cation-π interaction to nicotine.[23] Molecular dynamics simulations suggest that this interaction is discouraged when a Gly is present at this position.[23]

In the present study we find that the α1G153K mutation generically increases agonist affinity for the muscle-type receptor by 30−100-fold (**Table 3.1**). However, this mutation had little effect on the magnitudes of EC_{50} fold-shifts seen for ACh or choline in response to backbone mutation at γL119/δL121. Similarly, mutant cycle analysis of γL119Lah/δL121Lah and ACh/choline with the α1G153K mutation gave a coupling energy that was comparable to the value seen in the absence of the mutation (**Table 3.2**) A small increase in the fold-shift in EC_{50} was seen for nicotine and epibatidine, suggesting that the α1G153K mutation may have moderate effects on agonist binding to the γL119/δL121 residue for these agonists.

3.4 DISCUSSION

In recent years, the well-studied nicotinic pharmacophore has been mapped onto specific binding interactions in the nAChR. 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,[18, 20] backbone mutagenesis and mutant cycle analysis studies established a hydrogen bond between the pharmacophore acceptor (pyridine N of nicotine; carbonyl O of ACh) and the backbone NH of β2Leu119 in the α4β2 neuronal nAChR (analogous to the γL119/δL121 residue in the muscle-type receptor).[12] In the present work, we evaluate binding interactions to the

hydrogen bond acceptor of the pharmacophore in the pharmacologically distinct muscle-type nAChR.

The AChBP structures actually predict that the hydrogen bond to the hydrogen bond acceptor is mediated by a water molecule that hydrogen bonds to both the backbone NH of γL119/δL121 and the backbone CO of another residue in the complementary subunit, γN107/δN109. The backbone ester strategy employed here allows us to probe both components of the hydrogen bond system.

ACh and nicotine both show a strong hydrogen bonding interaction with the backbone NH of γL119/δL121 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 backbone ester mutation at γL119/δL121, more sensitive than it is to mutation of the corresponding residue in α4β2, where nicotine is a very potent agonist. Backbone mutation at γL119/δL121 also impacted ACh potency much more in the muscle-type receptor than in the α4β2 receptor. This may suggest that this hydrogen bond 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. For nicotine, this is consistent with the fact that it does not make a cation-π interaction to TrpB in the muscle-type receptor,[13] but does in α4β2.[15]

Given the strong interaction between nicotine and γL119/δL121, it is quite surprising that epibatidine is unresponsive to mutation at this site in the muscle-type receptor. Also unlike nicotine, epibatidine *does* make the cation-π interaction to TrpB in this receptor.[13] This suggests that perhaps epibatidine binds closer to TrpB than nicotine, and thus further from γL119/δL121. Despite the fact that they are very similar

structurally, it is clear that epibatidine and nicotine display differential binding preferences in the muscle-type nAChR.

We find that all agonists tested were unaffected by backbone mutation at γV108/δV110 in the muscle-type receptor, ruling out a functional role for a hydrogen bond to the backbone CO of γN107/δN109. Note that efforts to study this mutation in α4β2 were not successful for technical reasons, and so this is the first evaluation of this potential binding interaction. As noted above, many studies have shown that this type of backbone mutation can strongly impact the strength of a hydrogen bond to a backbone carbonyl. For example, when using this strategy to probe the hydrogen bond from the N^+H of agonists to the CO of TrpB in α4β2, the ester mutation caused increases in EC_{50} from 9-fold to 27-fold. Also, similar mutations have been shown to have significant impacts on the stabilities of both α-helices and β-sheets.[31, 38, 39] As such, our current data strongly suggest that γN107/δN109 does not play a significant role in agonist binding.

Our results thus find support for one, but not the other, of the two water-mediated hydrogen bonds predicted by the AChBP structures. Note that for either backbone ester mutation, the proposed water molecule could remain after mutation (coordinated by the backbone component that is not mutated), and so it was possible that neither mutation would have a large effect. However, this is not the case, as the 29-fold shift with ACh seen for the γL119Lah/δL121Lah mutation is quite large for this type of perturbation. Furthermore, there is a fundamental distinction between the two hydrogen bond interactions seen in AChBP. The backbone NH of γL119/δL121 can hydrogen bond *directly* to the hydrogen bond acceptor of agonists; the backbone CO of γN107/δN109 can only do so through an intermediary water. Perhaps this distinction rationalizes the

differing ways the two putative hydrogen bonds respond to our probes, including the possibility that the key water molecule seen in AChBP is not present in the nAChR. The backbone NH of γL119/δL121 would then interact directly with the hydrogen bond acceptor of agonists.

Earlier studies have shown that the identity of the residue at position 153 of the α subunit strongly impacts receptor function.[23] An α1G153K in the muscle-type receptor greatly increases nicotine potency, and it does so by facilitating a strong cation-π interaction to TrpB that is absent in the wild-type receptor.[15] We have now found that the α1G153K mutation does not have a substantial impact on the γL119/δL121 interaction. This is perhaps not surprising, given that the α1G153 residue is located in the principal component of the agonist binding site, while γL119/δL121 lies across the subunit interface in the complementary component of the binding site.

In summary, we have shown that ACh and nicotine both engage in a hydrogen bond to the complementary subunit residue γL119/δL121 in the muscle-type nAChR, but the nicotine analog epibatidine does not. In the α4β2 receptor all three agonists engage in this interaction, but the sensitivity of ACh and nicotine to backbone ester mutation at this residue is less than what is seen in the muscle-type receptor. We have also shown that the backbone CO of γN107/δN109, which is predicted by AChBP structures to participate in a water-mediated hydrogen bond with γL119/δL121, is not important for agonist-mediated activation of the muscle-type receptor. Introduction of the α1G153K mutation has only marginal effects on the γL119/δL121 hydrogen bond. Taken together, these data provide a clearer picture of the agonist binding mechanisms of the muscle-type nAChR

and highlight subtle variations in the mechanisms used by different receptor subtypes, which could offer new insight into the design of subtype-selective therapies.

3.5 EXPERIMENTAL SECTION

Mutagenesis. Nonsense suppression was performed using techniques described previously[40] on mouse muscle embryonic nAChR ($\alpha 1_2\beta 1\gamma\delta$) cDNA in the pAMV vector. For nonsense suppression experiments, a TAG (for mutation at γV108/δV110) or TGA stop codon (for mutation at γL119/δL121) 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.[36, 37] 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 and mRNA was prepared by *in vitro* transcription using the mMessage Machine T7 kit (Ambion).

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 or nonsense suppression experiments, respectively. 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.[40] For wild-type or conventional experiments, 1-2 ng of mRNA was injected per oocyte in a single 75 nL injection. For nonsense suppression experiments, 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). 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 hrs after injection for the wild-type/conventional or nonsense suppression experiments, respectively. Wild-type recovery control experiments (injection of tRNA appended to the natural amino acid) were preformed to evaluate the fidelity of the nonsense suppression experiments. Additional controls, injections of mRNA only and mRNA with 76-mer THG73, were also performed and gave minimal currents in electrophysiology experiments (~100 nA or less for controls compared to >>2 μA for nonsense suppression experiments).

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 instrument (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. 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. A detailed error analysis of nonsense suppression experiments shows that data are reproducible to ±50% in EC_{50}.[41]

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