CHAPTER 4: Stereochemical Preferences of the Nicotinic Receptor: Pharmacophore Binding Interactions of Epibatidine Enantiomers*

4.1 ABSTRACT

The nicotinic acetylcholine receptor is responsive to a number of small alkaloids, and not surprisingly, the prototype nicotine shows a strong bias for one drug enantiomer over the other. However, for the closely related and highly potent epibatidine, the two enantiomers are equipotent at the neuronal α4β2 receptor. Moreover, *N*-methylation of epibatidine negatively impacts the potency of just one enantiomer. To understand these observations, we sought to characterize the ligand binding mechanisms of the two enantiomers of epibatidine and their *N*-methyl derivatives. Using unnatural amino acid mutagenesis, we find that despite their equipotency, the enantiomers of epibatidine display striking differences in sensitivities to perturbations of key binding interactions, while their *N*-methyl derivatives are nearly identically sensitive to these perturbations. These data suggest that other non-covalent interactions may be important for defining epibatidine potency or that a combination of effects arising from agonist binding and receptor gating give rise to the observed potencies.

4.2 INTRODUCTION

Synaptic transmission in the central and peripheral nervous systems is mediated by nicotinic acetylcholine receptors (nAChRs).[1-3] nAChRs are ligand-gated ion channels that are activated by the neurotransmitter acetylcholine and also by nicotine and other

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* *This work was done in collaboration with Wesley Yu, a Caltech undergraduate student.*

agonists that adhere to the nicotinic pharmacophore. They are implicated in essential processes including learning, memory, and antinociception and are also therapeutic targets for many disorders and conditions including Alzheimer's disease, Parkinson's disease, schizophrenia, and nicotine addiction.[4, 5]

nAChRs are the prototype of a superfamily of structurally and functionally related neurotransmitter-gated ion channels called the Cys-loop (or pentameric) receptors. This group contains several other families of receptors, including the 5-HT_3 serotonin receptors; the $GABA_A$ and $GABA_C$ receptors; and glycine receptors. Each family member is a pentamer, composed of five subunits arranged around a central, ion-conducting pore. Individual subunits consist of a four-helix transmembrane domain that contains the ion channel gate, an N-terminal extracellular ligand-binding domain and a short extracellular C-terminus. There are 16 known mammalian nAChR subunits that give rise to >20 active nAChR subtypes in humans.[4, 6] nAChR subtypes are expressed in the peripheral nervous system at neuromuscular junctions (muscle-type receptors) and also in neurons of the central nervous system (neuronal receptors). The α4β2 receptor is the most abundant neuronal nAChR and the subtype most associated with nicotine addiction.[7-10] It is also the intended target of Chantix®, an established smoking cessation drug.[7]

In the α4β2 subtype, agonist binding occurs at the interface of adjacent principal (α4) and complementary (β2) subunits. Each subunit contributes several 'loop' segments that constitute the agonist binding site. The α4 subunit provides loops A, B and C, while the β2 subunit supplies loops D, E and F. Loops A, B, C and D contribute five highly conserved aromatic residues collectively referred to as the 'aromatic box' that are

important for agonist binding. Nicotinic agonists share a common pharmacophore, comprised of two essential components − a cationic N and a hydrogen bond acceptor (the CO of ACh or the pyridine N of nicotine) separated by an appropriate 'internitrogen' distance.[11, 12] The binding partners of the nicotinic pharmacophore have been identified by unnatural amino acid mutagenesis studies[13-16] and also by the structural characterization of the acetylcholine binding proteins (AChBPs),[17-22] which share ~25% sequence homology to the extracellular ligand binding domain of the nAChRs. The binding interactions of the pharmacophore are summarized in **Figure 4.1** using epibatidine, a structural analog of nicotine, as the model compound. In this model, the cationic center of the pharmacophore engages in a cation-π interaction to the aromatic box residue, TrpB. Agonists with protonatable nitrogens like nicotine and epibatidine (but not ACh) can also participate in a hydrogen bond to the backbone CO of TrpB. The second component of the pharmacophore, the hydrogen bond acceptor, makes a hydrogen bond to the backbone NH of L119 of the complementary β2 subunit. In the AChBP structure with nicotine bound,[17] this interaction is depicted as a water-mediated hydrogen bond, but the key structural water is not present in structures with carbamylcholine bound[17] or (+)-epibatidine bound.[18, 20] The overall binding model shown in **Figure 4.1** has been verified for several agonists including ACh, nicotine and (±)-epibatidine by mutagenesis studies in the α4β2 receptor.[14-16]

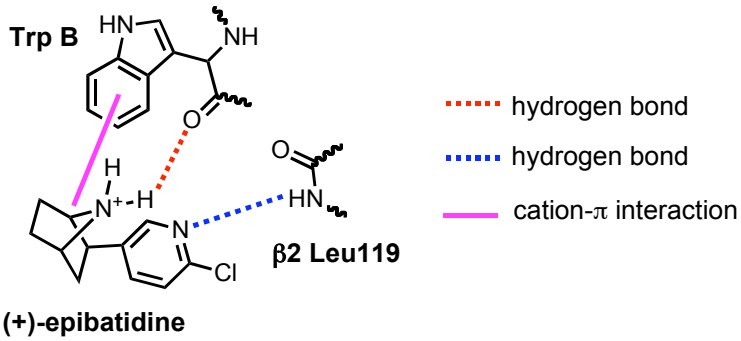

Figure 4.1. The binding interactions of the nicotinic pharmacophore shown for (+)-epibatidine. Note that the nicotine-bound AChBP crystal structure[17] also predicts a second hydrogen bond to the pharmacophore's hydrogen bond acceptor involving another residue in the complementary subunit (β2N107), however, this residue was shown to be unimportant for agonist binding in the muscle-type receptor as discussed in Chapter 3 of this thesis.

One puzzling aspect of nAChR pharmacology is that the two enantiomers of epibatidine (**Figure 4.2A**) are equipotent.[23] This is surprising given that the nAChR is a chiral molecule that ought to engage in some degree of chiral recognition. Indeed, this is the case for nicotine– the naturally occurring stereoisomer, *S*-(−)-nicotine, has a higher affinity for the nAChR and is also 10−100-fold more potent than its enantiomer.[24-26] Moreover, the molecular structures of epibatidine and nicotine are strikingly similar. Both adhere to the nicotinic pharmacophore with a pyridine N as the hydrogen bond acceptor and a cationic N that is part of a five-membered ring (epibatidine's five-membered ring is part of its azabicycloheptane structure), and so it is curious that the stereoisomers of the two agonists are received differently by the nAChRs.

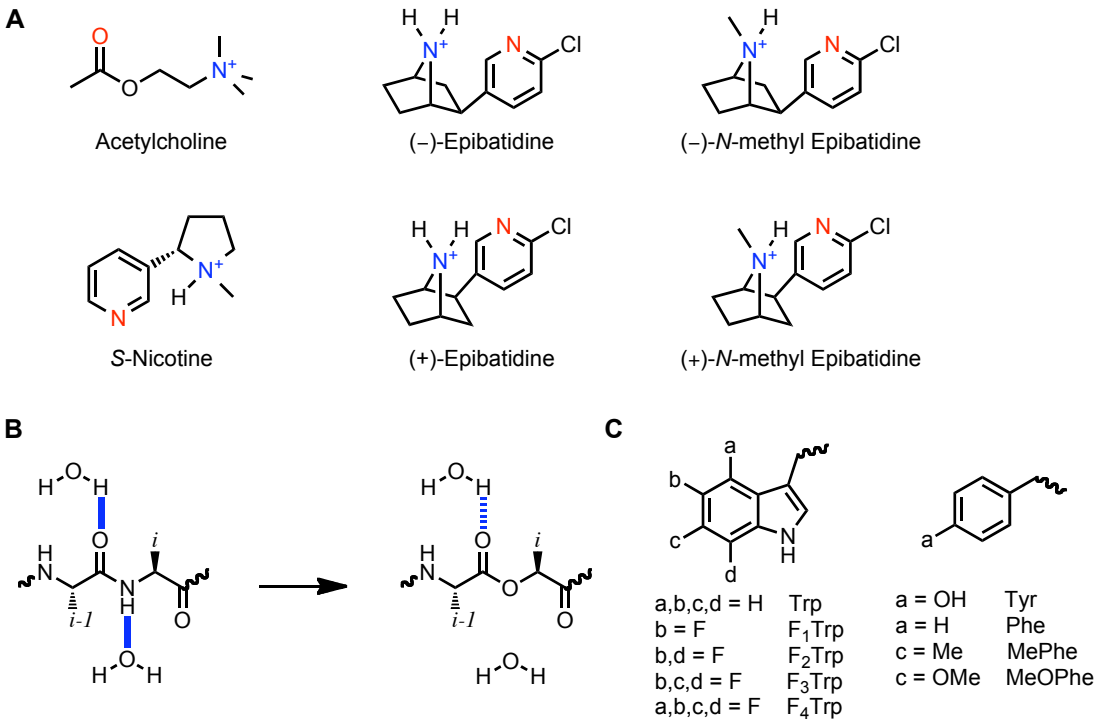

Figure 4.2. Agonists and unnatural amino acids used in this study. (A) Agonist structures. (B) Unnatural amino acids used to probe interactions to TrpB and TyrA. (C) The backbone ester strategy for probing hydrogen bonds to a protein backbone.

N-Methylation of the epibatidine enantiomers produces stereoisomers with different EC_{50} values (a measure of potency) at the α4β2 receptor.[23, 27] While the EC_{50} of (–)-*N*-methyl epibatidine is equivalent to the values seen for both enantiomers of the parent compound, the EC_{50} of (+)-*N*-methyl is ten-fold higher. We wondered whether this difference in EC_{50} was the result of a disruption to a hydrogen bond, possibly the hydrogen bond to the backbone CO of TrpB, that results from *N*-methylation of the (+)-epibatidine, but not its enantiomer.

Here, we use unnatural amino acid mutagenesis to characterize the strength of the pharmacophore binding interactions of epibatidine enantiomers and their *N*-methyl derivatives to determine whether established agonist binding mechanisms account for the observed similarities and differences in agonist potency. Surprisingly, we find that the

two enantiomers of epibatidine respond differently to mutagenesis studies of these interactions despite their equipotency, and the *N*-methyl enantiomers respond similarly to these mutations even though there is a ten-fold discrepancy in their potency at the wild-type α4β2 receptor. These data suggest that other factors could contribute to the observed potencies, which could include alternative binding interactions or gating effects.

4.3 RESULTS

4.3.1 General Strategy

These studies use nonsense suppression methodology to study the binding interactions of the pharmacophore for each enantiomer of epibatidine and *N*-methyl epibatidine in the α4β2 receptor expressed in *Xenopus* oocytes. The α4β2 subtype assembles into two viable pentameric stoichiometries, $(\alpha4)_2(\beta2)_3$ and $(\alpha4)_3(\beta2)_2$. This work focuses on the $(\alpha4)_2(\beta2)_3$ stoichiometry, which is more sensitive to nicotine[7-10] and is upregulated during chronic nicotine exposure.[28] In these studies, EC_{50}, the agonist concentration that elicits a half-maximal response (the midpoint of a dose-response curve), is used as a functional measure of agonist potency. EC_{50} is influenced by agonist binding and also by receptor gating (ion pore opening). Given the 60 Å distance between the agonist binding site and the channel gate, it is anticipated that mutations made to the residues studied here primarily affect agonist binding and not channel gating. This assumption is, in part, supported by single-channel studies of α4β2 that showed that mutations to TrpB had little impact on P_{open} (the probability that the channel is open),[14] suggesting that changes in EC_{50} that result from mutation of this residue are likely to primarily result from disruptions to agonist binding.

Each interaction to the nicotinic pharmacophore depicted in **Figure 4.1** can be readily probed by nonsense suppression methodology. Hydrogen bonds to protein backbones can be probed by replacing the residue that contributes the backbone NH with the analogous α-hydroxy acid (**Figure 4.2B**).[29-32] This converts the backbone amide to an ester and in doing so eliminates a hydrogen bond donor by replacing the backbone NH with an O. As such, we probe the hydrogen bond to the backbone NH of L119 by replacing this residue with the corresponding α-hydroxy acid (Lah; leucine, α-hydroxy). It is also well-established that the CO of an ester is a much poorer hydrogen bond acceptor than the CO associated with an amide, and so the hydrogen bond-accepting ability of the backbone CO (of the *i-1* residue) is also attenuated by backbone ester mutations. Therefore, we probe the hydrogen bond to the backbone CO of TrpB by replacing the *i+1* residue, Thr155, with its α-hydroxy analog. We consider the fold-shift in EC_{50} for a backbone ester mutation to be an indication of the strength of the hydrogen bond interaction being probed. We feel this is appropriate given the subtlety of these mutations (which convert a single backbone NH to an O) and the structural similarity of the agonists used in these studies.

To probe for the cation-π interaction, a series of fluorinated Trp analogs is incorporated at TrpB (**Figure 4.2C**).[33-35] Fluorination attenuates the cation binding ability of the Trp side chain in an additive fashion. A linear correlation between the EC_{50} and the cation-π binding ability of the side chain is indicative of cation-π interaction. In these studies we will be using the relative slope of these linear correlations (or 'fluorination plots') as an indication of the relative strength of a cation-π interaction. As

an alternative measure, the EC_{50} fold-shifts for each fluorinated mutant will also be compared.

4.3.2 EC_{50} values at the α4(L9'A)β2 receptor

Consistent with what has been reported for the wild-type α4β2 receptor,[27] we find that both enantiomers of epibatidine and (–)-*N*-methyl epibatidine are equipotent and the EC_{50} of (+)-*N*-methyl epibatidine is ~ten-fold higher than the other three compounds at the α4(L9'A)β2 receptor (**Table 4.1**). Mutagenesis studies of the three interactions of the pharmacophore are discussed below.

Table 4.1. EC_{50} and Hill coefficient values (± standard error of the mean) for epibatidine and *N*-methyl derivatives. Mutations identified as "Leu," "Thr," "Trp," and "Tyr" represent recovery of the wild type receptor by nonsense suppression.

Mutation	Agonist	EC_{50} (nM)	Hill
α4(L9'A)β2	(+)-Epi	0.87 ± 0.03	1.5 ± 0.1
	(−)-Epi	1.1 ± 0.04	1.6 ± 0.1
	(+)-Epi-Me	8.6 ± 0.5	1.7 ± 0.2
	(−)-Epi-Me	0.42 ± 0.04	1.5 ± 0.1
α4(L9'A)β2(L119Leu)	(+)-Epi	0.58 ± 0.04	1.3 ± 0.1
	(−)-Epi	0.73 ± 0.03	1.4 ± 0.1
	(+)-Epi-Me	8.9 ± 0.7	1.3 ± 0.1
	(−)-Epi-Me	0.42 ± 0.04	1.6 ± 0.1
α4(L9'A)β2(L119Lah)	(+)-Epi	2.7 ± 0.1	1.3 ± 0.1
	(−)-Epi	3.8 ± 0.2	1.1 ± 0.1
	(+)-Epi-Me	30 ± 2	1.4 ± 0.1
	(−)-Epi-Me	0.91 ± 0.06	1.9 ± 0.2
α4(L9'A/T155Thr)β2	(+)-Epi	0.89 ± 0.09	1.2 ± 0.1
	(−)-Epi	1.8 ± 0.07	1.5 ± 0.1
	(+)-Epi-Me	6.2 ± 0.4	1.4 ± 0.1
	(−)-Epi-Me	0.35 ± 0.02	1.6 ± 0.2
α4(L9'A/T155Tah)β2	(+)-Epi	4 ± 0.3	1.3 ± 0.1
	(−)-Epi	17 ± 0.6	1.4 ± 0.1
	(+)-Epi-Me	93 ± 7	1.5 ± 0.1
	(−)-Epi-Me	4.1 ± 0.3	1.3 ± 0.1
α4(L9'A/W154Trp)β2	(+)-Epi	0.92 ± 0.04	1.5 ± 0.1
	(−)-Epi	1.2 ± 0.04	1.7 ± 0.1
	(+)-Epi-Me	7.6 ± 0.5	1.3 ± 0.1
	(−)-Epi-Me	0.65 ± 0.08	1.5 ± 0.2
α4(L9'A/W154F_1Trp)/β2	(+)-Epi	1.8 ± 0.1	1.3 ± 0.1
	(−)-Epi	9.2 ± 0.4	1.5 ± 0.1
	(+)-Epi-Me	35 ± 2	1.3 ± 0.1
	(−)-Epi-Me	3.5 ± 0.3	1.3 ± 0.1
α4(L9'A/W154F_2Trp)/β2	(+)-Epi	2.3 ± 0.1	1.3 ± 0.1
	(−)-Epi	15 ± 0.6	1.3 ± 0.1
	(+)-Epi-Me	44 ± 3	1.2 ± 0.1
	(−)-Epi-Me	5.7 ± 0.7	1.2 ± 0.1
α4(L9'A/W154F_3Trp)/β2	(+)-Epi	16 ± 1	1.2 ± 0.1
	(−)-Epi	76 ± 1	1.3 ± 0.1
	(+)-Epi-Me	190 ± 10	1.6 ± 0.1
	(−)-Epi-Me	33 ± 2	1.4 ± 0.1
α4(L9'A/W154F_4Trp)/β2	(+)-Epi	20 ± 2	1.0 ± 0.1
	(−)-Epi	210 ± 7	1.3 ± 0.1
	(+)-Epi-Me	400 ± 20	1.2 ± 0.1
	(−)-Epi-Me	35 ± 3	1.2 ± 0.1
α4(L9'A/Y98Tyr)β2	(+)-Epi	0.64 ± 0.04	1.5 ± 0.1
	(−)-Epi	0.8 ± 0.05	1.4 ± 0.1
	(+)-Epi-Me	6.4 ± 0.2	1.3 ± 0.1
	(−)-Epi-Me	0.77 ± 0.2	1.0 ± 0.1
α4(L9'A/Y98MeOPhe)β2	(+)-Epi	9.2 ± 0.6	1.3 ± 0.1
	(−)-Epi	9.2 ± 0.5	1.5 ± 0.1
	(+)-Epi-Me	45 ± 2	1.4 ± 0.1
	(−)-Epi-Me	0.8 ± 0.1	1.9 ± 0.1
α4(L9'A/Y98MePhe)β2	(+)-Epi	42 ± 3	1.5 ± 0.1
	(−)-Epi	16 ± 1	1.3 ± 0.1
	(+)-Epi-Me	34 ± 2	1.3 ± 0.1
	(−)-Epi-Me	0.89 ± 0.09	2.0 ± 0.3
α4(L9'A/Y98Phe)β2	(+)-Epi	1.5 ± 0.1	1.4 ± 0.1
	(−)-Epi	40 ± 2	1.3 ± 0.1
	(+)-Epi-Me	66 ± 4	1.1 ± 0.1
	(−)-Epi-Me	0.82 ± 0.08	1.6 ± 0.1

4.3.3 Cation-π interaction to Trp B

All epibatidine isomers and derivatives were highly sensitive to fluorination of the TrpB side chain, but differences were seen in the magnitude of their sensitivities and in the slopes of their fluorination plots (**Figure 4.3** and **Table 4.2**). (+)-Epibatidine gave the smallest fluorination plot slope of any of the four epibatidine compounds. This slope was also smaller than the slopes seen for ACh and nicotine in previous studies.[14] In contrast, its enantiomer, (−)-epibatidine, gave the largest slope we have ever seen at the $(\alpha 4)_2(\beta 2)_3$ stoichiometry. This difference was not seen in the *N*-methyl enantiomers, which gave equivalent fluorination slopes.

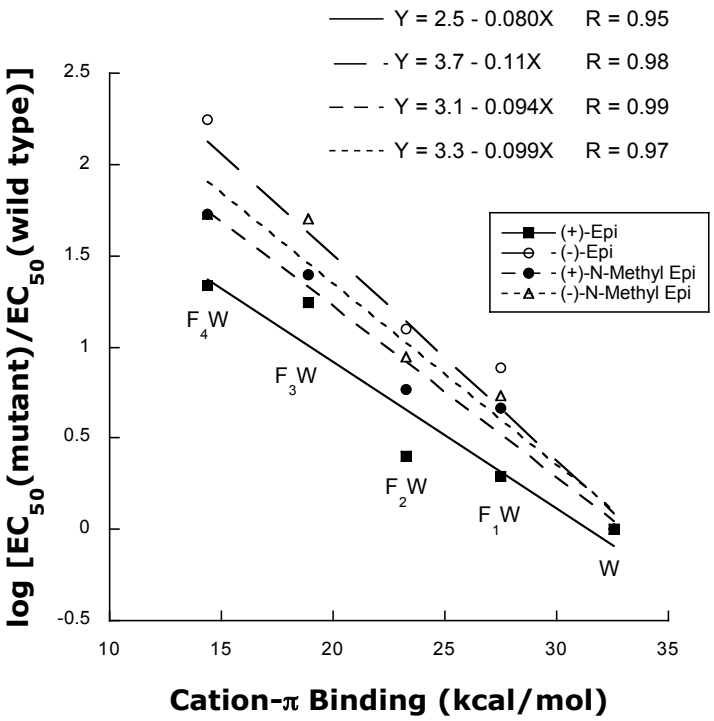

Figure 4.3. Fluorination plots of epibatidine compounds.

Table 4.2. EC_{50} fold-shifts and fluorination plot slopes for the epibatidine (Epi) compounds and also for ACh and nicotine (Nic). The data for ACh and nicotine are from previously published studies.[14, 15]

Mutation	(+)-Epi	(−)-Epi	(+)-*N*-methyl Epi	(−)-*N*-methyl Epi	ACh*	Nic*
			EC_{50} Fold-Shift			
α4(L9'A/W154F$_1$Trp)β2	2.0	7.7	4.6	5.4	4.3	2.9
α4(L9'A/W154F$_2$Trp)β2	2.5	13	5.8	8.8	4.5	3.6
α4(L9'A/W154F$_3$Trp)β2	17	63	25	51	30	13
α4(L9'A/W154F$_4$Trp)β2	22	180	53	54	65	47
α4(L9'A/T155Tah)β2	4.5	9.4	15	12	1.0	21
α4(L9'A)β2(L119Lah)	4.7	5.2	3.4	2.2	7.0	7.0
			Fluorination Plot Slope			
	0.080	0.11	0.094	0.099	0.10	0.089

Comparison of the magnitudes of the EC_{50} fold-shifts suggests a similar trend. The EC_{50} fold-shifts for (+)-epibatidine were significantly smaller than those seen for (–)-epibatidine, highlighted by the 22- vs. 180-fold-shift seen for F_4Trp. In contrast, the shifts for the *N*-methyl derivatives were relatively similar despite the ten-fold difference in wild-type EC_{50} for these enantiomers.

4.3.4 Hydrogen bond to the backbone CO of Trp B

The epibatidine compounds were also affected by backbone ester mutation of α4T155 (**Table 4.2**), suggesting that they each make a hydrogen bond to the backbone CO of TrpB. In general, the epibatidine compounds are less sensitive to this mutation than nicotine.[14] The EC_{50} fold-shift seen for (–)-epibatidine was double the shift seen for its enantiomer, which is surprising given that (–)-epibatidine was also more sensitive to fluorination at TrpB.

In general, the *N*-methyl derivatives were more sensitive to backbone ester mutation than the parent molecules. Similar to the trend seen in studies of the cation-π interaction, the *N*-methyl derivatives showed nearly identical shifts in EC_{50} in response to

backbone mutation despite the order-of-magnitude difference in their wild-type EC_{50} values.

4.3.5 Hydrogen bond to β2L119

The EC_{50} of each epibatidine compound was impacted by incorporation of an α-hydroxy acid at β2L119 (**Table 4.2**), although the magnitudes of the fold-shifts were smaller than what is seen for ACh and nicotine.[15] This suggests that the hydrogen bond to the backbone NH of this residue does not play a major role in binding epibatidine.

4.3.6 A hydrogen bond to TyrA?

A closer examination of the AChBP structure with (+)-epibatidine bound suggested that a second hydrogen bond might link the N^+H of epibatidine to the receptor.[18, 20] This hydrogen bond is to the phenol OH of the aromatic box residue TyrA. In the AChBP structure, (+)-epibatidine comes within 4 Å of this side chain.

It should be noted that we were skeptical as to whether we could obtain meaningful information from mutagenesis studies of TyrA, as this residue has been suggested to play a role in channel gating, although there is disagreement on this subject.[36, 37] The potency of ACh has been shown to be impacted by mutation of this residue to a Phe in *Torpedo*,[37, 38] muscle-type,[36] α7,[39] and α4β2[14] nAChRs. This is surprising given that the cationic N of ACh cannot serve as a hydrogen bond donor and therefore cannot participate in the hydrogen bond predicted for epibatidine by the AChBP structure. A single-channel study of the muscle-type receptor found that the predominant effect of the TyrA to Phe mutation is to alter the affinity of the agonist binding site, although a small, yet statistically significant, effect on P_{open} was seen.[40] Mutation of TyrA

to MePhe and MeOPhe in α4β2 also impacts the EC_{50} of ACh and nicotine, but to a lesser extent (**Table 4.3**).[14] It could be that mutation of the 4-position of the TyrA side chain generically impacts receptor function, possibly owing to its involvement in interactions with other residues of the protein that are important for protein structure/function or that, indeed, channel gating is effected by mutation of this residue. Given this history, it was anticipated that it would be difficult to tease out information about the predicted interaction to TyrA using EC_{50} comparisons. Nevertheless, we attempted to probe this interaction.

With respect to EC_{50} fold-shifts, one of the more surprising results from our studies of TyrA is that (–)-*N*-methyl epibatidine is completely insensitive to any of the mutations we tested (**Table 4.3**). This is particularly remarkable because it is the more potent enantiomer of the *N*-methyl derivatives and so might have been considered to be more likely to participate in the predicted hydrogen bond than its enantiomer (which *is* sensitive to mutation of TyrA). Importantly, these data also suggest that mutation of this residue does not generically impact receptor function. To our knowledge, (–)-*N*-methyl epibatidine is the only agonist that has been shown to be insensitive to mutation of TyrA.

Table 4.3. Relative efficacy values and EC_{50} fold-shifts resulting from mutation of TyrA (Y98) for the epibatidine (Epi) compounds, ACh and nicotine (Nic). The data for ACh and nicotine are from previously published studies.[14] The relative efficacy of an agonist is defined as the ratio: I_{max} of agonist at a saturating concentration / I_{max} of acetylcholine at a saturating concentration. By definition, the relative efficacy of ACh is 1.

Mutation	(+)-Epi	(−)-Epi	(+)-*N*-methyl Epi	(−)-*N*-methyl Epi	ACh*	Nic*
			EC_{50} Fold-Shift			
α4(L9'A/Y98MeOPhe)β2	14	12	7.0	1.0	7.4	5.0
α4(L9'A/Y98MePhe)β2	66	20	5.3	1.2	8.7	3.5
α4(L9'A/Y98Phe)β2	2.3	50	10	1.1	27	10
Relative Efficacy WT	0.3	0.7	0.5	0.6	[1]	0.3
Relative Efficacy Y98F	7	0.7	2	4	[1]	0.2

The EC_{50} values of the other three epibatidine compounds were sensitive to TyrA mutations. While the magnitude of the impact on EC_{50} varies between these compounds, no obvious trends are seen. (+)-Epibatidine is more sensitive to the MeOPhe and MePhe mutations than to the Tyr to Phe mutation; (–)-Epibatidine is most sensitive to the Tyr to Phe mutation; and (+)-Epibatidine is generically sensitive to mutation of this side chain.

A comparison of the relative efficacy of each agonist at the α4(L9'A)β2 receptor with and without the TyrA to Phe mutation shows dramatic differences for three of the epibatidine compounds, but no major difference for nicotine or (−)-epibatidine (**Table 4.3**). It is again interesting that the efficacies of the *N*-methyl derivative enantiomers are similarly affected by mutation, but the parent enantiomers display striking differences.

Efficacy is a measure of the agonist's ability to gate the channel, and so it is quite possible that the observed effects on efficacy and EC_{50} are primarily or partially the result of effects on channel gating. As such, it would be valuable to perform detailed single-channel studies to determine whether the observed impacts on agonist potency and efficacy are the result of alterations in channel gating, agonist binding or both.

4.4 DISCUSSION

Epibatidine was first isolated in 1974 by Daly and co-workers from the skin of the Ecuadorian 'poison dart' frog *Epipedobates tricolor*, which secretes the compound as a poison in the skin on their backs for protection from predators.[41] Daly demonstrated that epibatidine is a powerful analgesic, ~200 times more potent than morphine, but does not target opioid receptors. The latter finding generated excitement, because it meant that epibatidine was less likely to be addictive, but formidable toxic side effects have prevented its use as a therapeutic.[23] Instead, epibatidine is often used as a structural

starting point for the development of new pharmaceuticals.[23] Due to its high uptake and slow clearance from mouse brains, epibatidine has also been used as a parent structure for the development of radiotracers for *in vivo* labeling of nAChRs.[42, 43]

The analgesic properties of epibatidine were shown to be mediated by the nAChR.[23] Indeed, epibatidine is a potent agonist of the nAChR, activating the receptors at lower concentrations than nicotine (~100-fold lower EC_{50} than nicotine). Although the two molecules share a common pharmacophore and a remarkably similar structural layout, striking differences are seen in the potencies of their enantiomers at the nAChRs. *R*-(+)-nicotine has a 10−100-fold higher EC_{50} than its enantiomer at nAChRs,[24-26] but the enantiomers of epibatidine are equipotent. The equipotency of the epibatidine enantiomers is puzzling, given that the nAChR is a chiral molecule that should preferentially bind to one enantiomer of a substrate.

We sought to probe the pharmacophore binding interactions of the two enantiomers of epibatidine in α4β2 in the hope of gaining a better understanding of the origin of their equipotency. We anticipated that (+)-epibatidine and (–)-epibatidine would behave in a similar fashion to perturbation of the three pharmacophore interactions, or that a compensatory relationship would be active. Consistent with expectation, we found that both enantiomers behaved identically to perturbation of the hydrogen bond to the complementary subunit, but this interaction does not appear to be very important for epibatidine binding in general. We also found that (−)-epibatidine is much more sensitive to perturbation of both TrpB interactions than its enantiomer– a surprising result given the equipotency of the two agonists.

N-Methylation of epibatidine has been shown to have a relatively dramatic effect on the potency of one of the epibatidine enantiomers. While (−)-*N*-methyl epibatidine shares the same potency as the parent compounds, its enantiomer has a ten-fold higher EC_{50}. To explain this discrepancy in EC_{50}, we wondered whether one of the interactions of the pharmacophore was disrupted by methylation of one enantiomer, but not the other. Surprisingly, we found that both enantiomers of the *N*-methyl derivative are nearly identically sensitive to perturbation of the three interactions. Thus, our data concerning the interactions of the pharmacophore do not account for the equipotency of epibatidine enantiomers or the discrepancy in EC_{50} seen for the *N*-methyl derivatives, but rather suggests that an alternative explanation is viable.

It could be that additional noncovalent interactions compensate for the stronger cation-π and hydrogen bond interactions seen for (–)-epibatidine and that disruption of these same or other interactions are responsible for the discrepancy in *N*-methyl derivative EC_{50} values. The AChBP structure with (+)-epibatidine bound suggests several possible interactions, including polar contacts between the pyridine ring Cl and residues in the complementary subunit of the protein as well as a second hydrogen bond to the N^+H of the pharmacophore.[18, 20] We anticipate that the predicted interactions with the Cl are unlikely to account for the large discrepancies in sensitivities we observe, because an epibatidine derivative lacking the Cl ((±)-norchloroepibatidine) has only minimally perturbed affinity and potency relative to the parent agonist.[44] The second predicted hydrogen bond involving the N^+H of the pharmacophore is to TyrA, one of the five aromatic box residues that are conserved across the Cys-loop family. This residue has played a complicated role in nAChR research.[36, 37, 40] ACh cannot serve as a hydrogen

bond donor and therefore cannot participate in the proposed interaction to TyrA, yet its EC_{50} is still very sensitive to mutation of this residue. Furthermore, single molecule studies suggest that mutation of this residue primarily effects agonist binding and not channel gating although small effects to P_{open} are seen for ACh.[40]

Our studies of this residue in the present context did not lead to any clear explanations for the potency of epibatidine and its derivatives, but they did show that the potency of (−)-*N*-methyl epibatidine is completely insensitive to mutation of TyrA, suggesting that mutations at this site do not generically affect agonist potency. While no measureable impact on the EC_{50} of (−)-*N*-methyl epibatidine was observed, the relative efficacy of this agonist was impacted by the TyrA to Phe mutation as were the relative efficacies of (+)-epibatidine and (+)-*N*-methyl epibatidine. Note that we are reporting *relative* efficacy values that are referenced to the I_{max} elicited by ACh. It is possible that the efficacy of ACh is also affected by the TyrA to Phe mutation, but this is probably not the case as the relative efficacies of (−)-epibatidine and nicotine are not affected by this mutation. Since efficacy is a measure of the ability of the agonist to induce channel opening, it is likely that mutations to TyrA may affect channel gating for some, if not all, agonists. As such, a single-channel kinetic study could be useful in understanding the effects of mutations to TyrA.

An alternative explanation for the observed agonist potencies is that a collection of hydrophobic interactions account for the observed similarities and differences in agonist potencies and sensitivities to mutations of the pharmacophore binding residues. Recall that the structure of epibatidine includes an azabicycloheptane structure, which could present a large hydrophobic surface area to the nAChR that might be more

accessible in the (+) enantiomer (which makes a weaker interactions with TrpB). The discrepancy in EC_{50} of the *N*-methyl derivatives could also be the consequence of steric effects that are introduced by methylation of one enantiomer but not the other. Given that EC_{50} is influenced by agonist binding and receptor gating, it is also possible that a combination of gating and binding effects coincidentally give rise to equipotency in one scenario and differences in potency in the other and also to the observed differences in sensitivities to perturbation of the pharmacophore interactions.

In summary, we have used nonsense suppression to evaluate the pharmacophore binding interactions of the enantiomers of epibatidine and *N*-methyl epibatidine to account for similarities and differences seen in their EC_{50} values at the neuronal α4β2 receptor. We find that all agonists participate in the pharmacophore interactions − a cation-π interaction to Trp B, a hydrogen bond to the backbone CO of TrpB, and a hydrogen bond to the backbone NH of L119 of the complementary subunit. However, we see surprising differences and similarities in their sensitivities to perturbations of these interactions that do not account for their respective EC_{50} values. Future work will seek to identify additional noncovalent interactions that could be responsible for the observed potencies of the four epibatidine compounds. It is also likely that a detailed kinetic analysis of the mutations used to probe these interactions would be valuable in uncovering this mystery.

4.5 EXPERIMENTAL SECTION

Nonsense suppression methodology was used to introduce unnatural amino acids and α-hydroxy acids site specifically in the α4β2 receptor.[45] Unnatural mutations were introduced to rat α4 and β2 cDNA in the pAMV vector by the standard Stratagene

QuickChange protocol, using a TAG codon for mutations to the α4 subunit and a TGA codon for mutations to β2. Mutations were verified by sequencing. cDNA was linearized with the restriction enzyme Not 1 and mRNA was prepared by *in vitro* transcription using the mMessage Machine T7 kit (Ambion). The α4 subunit contained the L9'A background mutation, which is known to increase receptor expression and sensitivity to agonists without affecting other aspects of receptor pharmacology.[46] The same mutation was used in our previous studies of the pharmacophore binding interactions in the α4β2 receptor.[14, 15]

Stage V-VI *Xenopus laevis* oocytes were injected with mRNA in a 1:1, 3:1 or 1:20 ratio of α4L9'A:β2 for wild-type experiments, nonsense suppression experiments in α4 or nonsense suppression experiments in β2, respectively. α-Hydroxy or amino acids were chemically acylated to the dinucleotide dCA and enzymatically ligated to the truncated 74-nucleotide THG73 or TQOpS' tRNA as previously described[45] for nonsense suppression experiments in α4 or β2, respectively. For nonsense suppression experiments, each cell was injected with 75 nL of a 1:1 mixture of mRNA (20-25 ng of total mRNA): tRNA (20-30 ng) while a 75 nL injection of 10 ng of mRNA was used for wild-type experiments. Injected oocytes were incubated for 24-48 hrs at 18 °C before electrophysiology recordings. Several control experiments were conducted to evaluate the fidelity of the nonsense suppression experiments, which included wild-type recovery experiments (injection of tRNA appended to the natural amino acid) and also injection of mRNA only or mRNA with unacylated suppressor tRNA. Negligible currents were seen for these controls.

Electrophysiology experiments were performed 24-48 hrs after injection using the OpusXpress 6000A instrument (Axon Instruments) in two-electrode voltage clamp mode with a holding potential of −60 mV. Ca^{2+} free ND96 solution was used as the running buffer (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.5). During electrophysiology recordings, the first 8 agonist doses (lowest concentrations) were applied for 90 seconds with a 116 s wash with running buffer, while the remaining doses were applied for 15 s with a 116 s wash. Dose-response data were obtained for ≥8 agonist concentrations on ≥6 cells. All EC_{50} and Hill coefficient values were obtained by fitting dose-response relations to the Hill equation and are reported as averages ± standard error of the fit. A detailed error analysis of nonsense suppression experiments reveals data are reproducible in EC_{50} to ±50%.[47] The stoichiometry of each mutant was verified by voltage jump experiments as described previously.[14]

Preparation of epibatidine and *N*-methyl enantiomers. 40 mg of (±)-epibatidine (Tocris) was separated by chiral HPLC using an Astec ChirobioticT column analytical column (Sigma Aldrich) with a 1.5 mL flow rate and 0.6% TEA and 0.4% AcOH in methanol as the mobile phase. Two fractions were collected with enantiomeric excess values of >99%. The fractions were concentrated to a 10 mL volume and 15 mL of 2M NaOH was added. The organic layer was extracted with CH_2Cl_2 (4×) and concentrated to afford pale yellow powders. Fraction 1 ((+)-epibatidine): $[\alpha]^{20}_D$ = + 7.0° (c = 1, $CHCl_3$). Fraction 2 ((–)-epibatidine): $[\alpha]^{20}_D$ = − 6.5° (c = 1, $CHCl_3$). NMR spectra before and after separation were identical and are consistent with previously reported data.[48]

To prepare the *N*-methyl derivatives, 10 mg of each enantiomer (0.048 mmol) was added to a separate two-neck, 25 mL round-bottom flask equipped with a reflux

condenser. To this was added 3 mL of formic acid and 1.5 mL of 37 wt% formaldehyde (in H_2O). The mixture was stirred and heated to reflux at 80 °C for 7 hrs. The solution was cooled to room temperature and made basic (pH 12) by the addition of 2M NaOH. The organics were extracted with CH_2Cl_2 (3×), washed with brine, dried over Na_2SO_4 and concentrated. The resulting oil was purified by flash column chromatography on silica gel (7% methanol in CH_2Cl_2). R_f = 0.34. NMR spectra are consistent with previously reported data.[42] Yield of (+)-*N*-methyl epibatidine: >90%, 10 mg. $[\alpha]^{20}_{D} = +120°$ (c = 1, $CHCl_3$). Yield of (–)-*N*-methyl epibatidine: >90%, 10 mg. $[\alpha]^{20}_{D} = -120°$ (c = 1, $CHCl_3$).

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