Chapter 3

Thinking Outside the Box: Probing the Functional Importance of Second Shell nAChR Binding Site Residues

3.1 INTRODUCTION

Nicotinic acetylcholine receptors (nAChR) are the most extensively studied members of the Cys-loop family of ligand gated ion channels (LGIC). These receptors are important in Alzheimer's disease, Schizophrenia, drug addiction, and learning and memory.[1,2] Through the binding of small molecule neurotransmitters, these transmembrane proteins undergo a conformational change that allows the protein to pass ions across the impermeable cell membrane. The embryonic mouse muscle nAChR is composed of five subunits, $(\alpha_1)_2\beta_1\gamma\delta$. Each subunit contains an extracellular ligand-binding domain at the N-terminus and four transmembrane domains (TM1-4). The two agonist-binding sites at the α/γ and α/δ interfaces are defined by a series of conserved aromatic residues.

In the past 5 years, the discovery and crystallization of the acetylcholine-binding protein (AChBP)[3,4] has significantly expanded knowledge of the nAChR ligand-binding domain (**Figure 3.1A**). AChBP is a homopentamer isolated from the snail *Lymnaea stagnalis* and it shares approximately 20% sequence homology with the nAChR extracellular ligand-binding domain. Sixma and co-workers have published a series of AChBP crystal structures: an initial 2.7 Å structure of AChBP,[4] a 2.2 Å nicotine-bound AChBP structure,[5] and a 2.5 Å carbamylcholine-bound AChBP structure.[6] This crystallographic information provides a model for studies examining nAChR ligand-receptor interactions and for drug discovery.

Because AChBP is not a neuroreceptor, chemical scale investigations have been crucial in identifying mechanistically significant drug-receptor interactions.[7,8] Consistent with early biochemical studies,[9,10] the x-ray structure of AChBP revealed a box-shaped agonist-binding site lined with a series of highly conserved aromatic amino acids.[4] The carbamylcholine bound AChBP structure[5] confirmed previous experimental studies that identified a stabilizing cation-π interaction between Trp α149 (αW149) and the quaternary ammonium of ACh.[11] Information from the nicotine bound AChBP structure suggested nicotine-like agonists, nicotine and epibatidine, could also be involved in a cation-π interaction with αW149.[5] Chemical scale investigations proved that this interaction is *not* important for nicotine binding,[8] but is important for epibatidine binding.[7] In addition, the nicotine-bound AChBP proposed a hydrogen bond between the protonated amine of nicotine and the backbone carbonyl of αW149 at the agonist-binding site.[5] The importance of this proposed hydrogen bond for nicotine and epibatidine binding was confirmed by chemical scale investigations by incorporating an α hydroxy acid at this muscle nAChR site.[7] These studies demonstrate the necessity of chemical scale investigations to probe the functional importance of the information revealed from the AChBP crystal structures.

Part A

Probing the Role of Highly Conserved Asp α89 in nAChR Function

The AChBP crystallographic data also proposed a structural role for Asp α89 (αD89), a highly conserved residue in the shell of amino acids immediately surrounding the agonist binding box.[4,5] Structural studies have implicated five separate hydrogen bonds, four involving the side chain of αD89 and one involving the backbone carbonyl (**Figure 3.1**). This intriguing network of hydrogen-bonding interactions appears well positioned to influence receptor function.

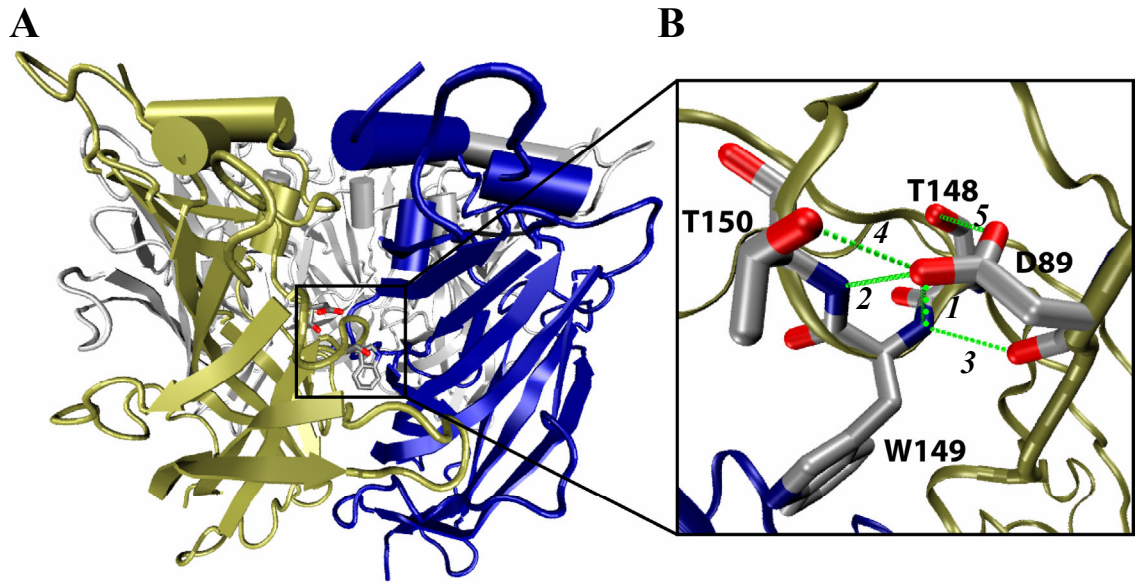

Figure 3.1. AChBP Crystal Structure. **A**) Crystal structure of AChBP in the carbamylcholine bound state. The subunit containing the principal binding site face is shown in gold. The subunit containing the secondary binding site face is shown in blue. **B**) The αD89 hydrogen bond network with backbone amides flanking αW149 and side chains of αT148 and αT150. Muscle type nAChR numbering is indicated.

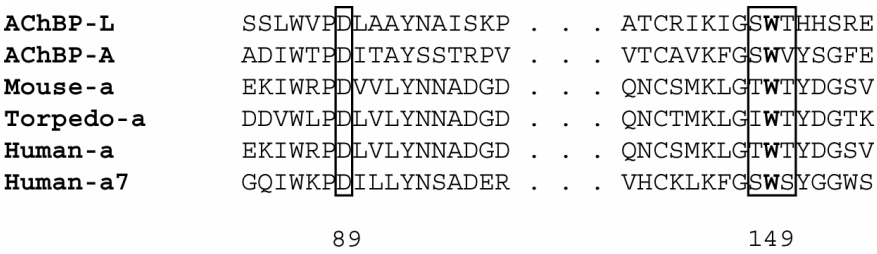

Figure 3.2. nAChR Sequence Alignment Near αD89.

Recently, Sine and co-workers studied mutations that eliminate one or two hydrogen bonds between the αD89 side chain and the αT148 and αT150 side chains, interactions 4 and 5, respectively.[12] These mutations displayed near wild-type ACh activity. Thus, the removal of one or two hydrogen bonds at these positions is tolerated for ACh. Instead, researchers inferred that hydrogen bonds between the side chain of αD89 and the backbone amides of αW149 and α150 must be important.[2] Studies further probing the importance of these proposed backbone hydrogen bonds are not possible through conventional mutagenesis.

To understand the structural importance of αD89, Sine and co-workers mutated αD89 to several residues, including Glu, Asn, and Thr.[2] These studies reveal that the side chain mutation αD89N, neutralizing the negative charge and introducing an electrostatic clash, dramatically impaired the kinetics of ACh binding. A similar neutralizing mutation, αD89T, also impaired channel function. On the other hand, a mutation extending the side chain length by one carbon and preserving the negative charge, αD89E, only slightly decreased ACh activity. To further explore the importance of charge in this region, a negative charge was introduced at the neighboring residues, αT148D and αT150D, in

attempt to recover the wild-type binding kinetics lost with the αD89N mutation. The double mutant αT148D/αD89N retrieved most of the wild-type binding kinetics, while αT150D/αD89N only recovered some. From these studies, researchers concluded that a negative charge is important at either αT148 or αD89 for near wild-type channel function.

The current study aimed to achieve two goals. First, we wished to evaluate the importance of the hydrogen bond network between αD89 and the amide backbone NHs at αW149 and αT150 in stabilizing the nAChR binding site. Second, we aimed to understand the structural requirements of the αD89 side chain for proper nAChR function. We examined the impact of these interactions on the activity of ACh, nicotine and epibatidine. To address these questions, the current study performed chemical scale investigations by utilizing *in vivo* nonsense suppression to incorporate unnatural amino acids in this region. This method offers a unique and powerful tool to subtly alter the properties of the protein backbone and amino acid side chains, otherwise unavailable with conventional mutagenesis.

α-hydroxy acids were incorporated at αW149 and αT150 to examine the importance of the hydrogen bond network between αD89 and the amide backbones surrounding αW149, interactions *1*, *2*, and *3*. By incorporating an α-hydroxy acid into a protein, the amide backbone is converted into an ester backbone, thereby eliminating a hydrogen bond donor and replacing it with an acceptor. The current study incorporated α-hydroxy tryptophan at position αW149 (αW149Wah) or α hydroxy threonine at position αT150 (αT150Tah) to determine the importance of interactions *1*, *2*, and *3*.

Mutations were performed at αD89 to determine the importance of the side chain on nAChR function. We incorporated a Glu mutation that extends the side chain of αD89, αD89E, and an Asn mutation that incorporates an amide side chain rather than the native carboxylate side chain at αD89, αD89N. Finally, we incorporated a neutral analogue, nitro-homoalanine (Nha) at αD89, an unnatural amino acid that is isosteric and isoelectronic with Glu but that lacks a negative charge. This analogue tests the importance of charge and hydrogen bond accepting ability of αD89, by neutralizing the charge and weakening the hydrogen bond accepting ability. Finally, double mutations αD89N/αW149Wah or αD89N/αT150Tah were evaluated in efforts to retrieve the loss of activity observed in αD89N single mutants.

3.2 RESULTS

Unnatural amino acids were incorporated into the nAChR using *in vivo* nonsense suppression methods. The structures of ACh, nicotine, and epibatidine are presented in **Figure 3.3.** The mutant receptor was evaluated using electrophysiology.[13] When studying weak agonists and/or receptors with diminished binding capability, it is necessary to introduce a Leu-to-Ser mutation at a site known as 9' in the second transmembrane region of the β subunit.[7,8] This 9' site in the β subunit is almost 50 Å from the binding site, and previous work has shown that a L9'S mutation lowers the effective concentration at half maximal response (EC_{50}) by a factor of roughly 40.[8,14] Results from earlier studies[8,14] and data reported below demonstrate that trends in EC_{50} values are not perturbed by L9'S mutations. Studies measuring nicotine EC_{50} for the αD89N/αT150Tah mutant required the introduction of a second Leu-to-Ser mutation at a

site known as 9'. This second 9' mutant exists in the second transmembrane region of the γ subunit. In addition, the alpha subunits contain an HA epitope between M3-M4. Control experiments show a negligible effect of this epitope tag on EC_{50}.[7] It should be noted that the EC_{50} value is not a binding constant, but a composite of equilibria for both binding and gating.

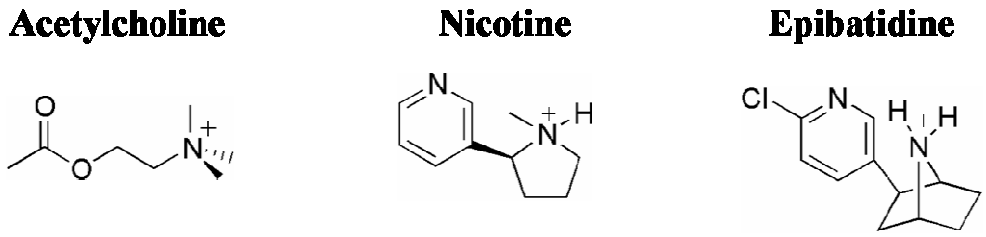

Figure 3.3 Chemical Structures of Agonists.

Single Hydrogen Bonds with αD89 Are Not Critical

The backbone amide protons αW149 and αT150 appear to help stabilize this region of the nAChR agonist-binding site by contributing to a hydrogen bond network with αD89. To evaluate this possibility, each backbone amide in this region was mutated to an ester, thereby eliminating one, or two, of the hydrogen bond donating partners with αD89. Ester mutations at αT150 and αW149 were performed by incorporating α-hydroxythreonine and α-hydroxytryptophan single mutants, αT150Tah and αW149Wah, respectively. The impact of these mutations was studied in the presence of ACh, epibatidine, and nicotine.

The results from incorporation of αW149Wah and αT150Tah are shown in **Table 3.1**. The αW149Wah mutant, eliminating interactions *1* and *3*, is tolerated for ACh and

nicotine, producing a 0.97 and 1.3-fold change in EC_{50}, respectively. Elimination of interactions *1* and *3* leads to a modest 2.7-fold *increase* in epibatidine EC_{50}. The αT150Tah mutant, eliminating interaction *2*, produces larger effects than the αW149Wah mutant, and, as discussed elsewhere,[7] part of the reason for this effect is the favorable interaction between the carbonyl of αW149 and the agonists nicotine and epibatidine. Therefore the trends in αT150Tah must also account for these interactions in addition to the hydrogen-bonding interactions with αD89. On balance, though, our results indicate that no single hydrogen bond within the network around αD89 is especially critical to receptor function.

Table 3.1 Mutations Testing H-bond Network[a]

Agonist	Wt[b]	α149Wah	α150Tah[b]
ACh	0.83 ± 0.04	0.81 ± 0.03	0.25 ± 0.01
Nic	57 ± 2	73 ± 2	92 ± 4
Epi	0.60 ± 0.04	1.6 ± 0.1	2.2 ± 0.2

[a] EC_{50} (μM) ± standard error of the mean. The receptor has a Leu9'Ser mutation in M2 of the β subunit. [b] Data reported previously.[7]

Role of Highly Conserved αD89

The highly conserved residue, αD89, has been proposed to play a structural role in shaping the nAChR agonist-binding site. To determine the importance of this residue on channel function, three mutations at αD89 were examined: Glu, Asn, and nitro-homoalannine. The results of these studies are reported in **Table 3.2**. The αD89E mutation, introducing an extended side chain, resulted in a 4.1-fold *increase* in ACh and

a 4-fold *increase* in epibatidine EC_{50} in comparison to wild-type receptors. The αD89E mutant resembles wild-type channels for nicotine. Thus, the extended side chain of αD89E slightly impairs ACh and epibatidine function and has little effect on nicotine function. The αD89N mutation, neutralizing the negative charge and introducing an electrostatic clash with adjacent amide backbone NHs, dramatically *increases* EC_{50} for ACh by 23-fold, nicotine by 28-fold, and epibatidine by 22-fold compared to wild-type receptors. These changes in EC_{50} values correspond to almost 2 kcal/mol energetically.

To understand the importance of charge and hydrogen bond accepting ability at αD89, the unnatural amino acid, nitro-homoalanine (Nha), was synthesized and incorporated at nAChR αD89 (αD89Nha). A nitro group is isosteric and isoelectronic with a carboxylate, but it lacks the negative charge. Sterically, Nha is equivalent to Glu, not Asp, but synthetic difficulties preclude the incorporation of the Asp analogue. Therefore, it is relevant to study the effects of αD89Nha on channel function in comparison to the Glu mutation, αD89E, with equivalent side chain length. The αD89Nha mutation resulted in a 4.7-fold *increase* in ACh EC_{50}, a 4.6-fold *increase* in epibatidine EC_{50}, and a 4.6-fold *increase* in nicotine EC_{50} compared to αD89E. These changes in EC_{50} values correspond to less than 1 kcal/mol. Thus, a modest decrease in nAChR activity was observed for all three agonists in the presence of αD89Nha.

Table 3.2 Mutations at αD89[a]

Agonist	Wt[b]	αD89E	αD89N	αD89Nha
ACh	0.83 ± 0.04	3.4 ± 0.3	19 ± 1	16 ± 0.9
Nic	57 ± 2	59 ± 6	1600[c]	270 ± 60
Epi	0.60 ± 0.04	2.4 ± 0.1	13 ± 1	11 ± 1

[a] EC_{50} (μM) ± standard error of the mean. The receptor has a Leu9'Ser mutation in M2 of the β subunit. [b] Data reported previously.[7] [c] This receptor also contains a Leu9'Ser mutation in M2 of the γ subunit, the value corrected for ease of comparison

αD89 Plays a Structural Role in Positioning αW149 Binding Site Backbone

An interesting observation is that αD89N dramatically impairs channel function, more so than any other mutation studied here. αD89N neutralizes the side chain and introduces an electrostatic clash with nearby backbone amides as shown in **Figure 3.4**. In an attempt to relieve the electrostatic clash, we examined the double mutants αD89N/αW149Wah and αD89N/αT150Tah. The results of these mutations in the presence of ACh, nicotine, and epibatidine studies are shown in **Table 3.3**.

Table 3.3 Understanding αD89N Mutation[a]

Agonist	Wt[b]	αD89N	αD89N, α150Tah	αD89N, α150Wah
ACh	0.83 ± 0.04	19 ± 1	15 ± 1	2.2 ± 0.1
Nic	57 ± 2	1600[c]	26 ± 1	~40[d]
Epi	0.60 ± 0.04	13 ± 1	2.9 ± 0.3	0.76 ± 0.05

[a] EC_{50} (μM) ± standard error of the mean. The receptor has a Leu9'Ser mutation in M2 of the β subunit. [b] Data reported previously.[7] [c] This receptor also contains a Leu9'Ser mutation in M2 of the γ subunit, the value corrected for ease of comparison. [d] Estimated value, studies are underway.

The αD89N/αW149Wah double mutation retrieves near wild-type activity for all three agonists. The electrophysiology traces for ACh and epibatidine are shown in **Figure 3.5.** The αW150Wah/αD89N double mutation leads to a ~1.4-fold *decrease* in nicotine EC_{50}, a 1.3-fold *increase* in epibatidine EC_{50}, and a 2.7-fold *increase* in ACh EC_{50} compared to wild-type receptors. The electrostatic clash between the αD89N amide side chain and the NH of αW149 was relieved upon incorporation of the ester, αW149Wah, in the presence of αD89N. The αD89N/αT150Tah double mutation, on the other hand only retrieves near wild-type activity for nicotine. Only a 2.2 fold *decrease* in nicotine EC_{50} is observed for the double mutation compared to wild-type receptors. In contrast, the αT150Tah/αD89N does not retrieve activity with potent agonists ACh and epibatidine where a 4.8-fold *increase* in epibatidine EC_{50} and an 18-fold *increase* in ACh EC_{50} are observed compared to wild-type receptors. Thus, the αD89N/αT149Tah mutation is only able to retrieve wild-type activity for weak agonist nicotine, but not for potent agonists, acetylcholine, and epibatidine.

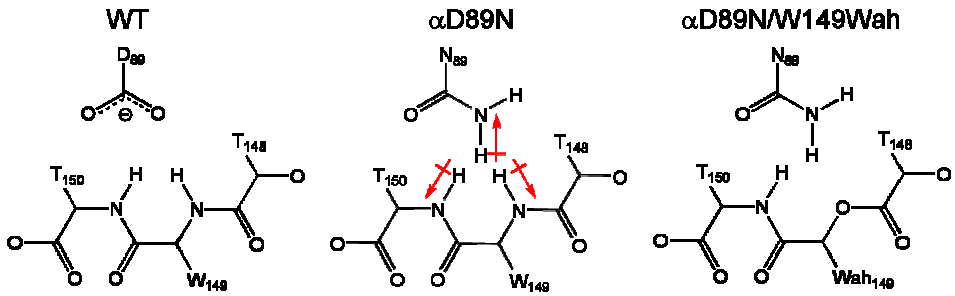

Figure 3.4. Electrostatic Clash at αD89N. Chemical structures of the proposed αD89 network. The red arrows represent the $N^{\delta-}$–$H^{\delta+}$ dipole involved in the electrostatic clash with αD89N. This clash is relieved with the αD89N/αW149Wah double mutant.

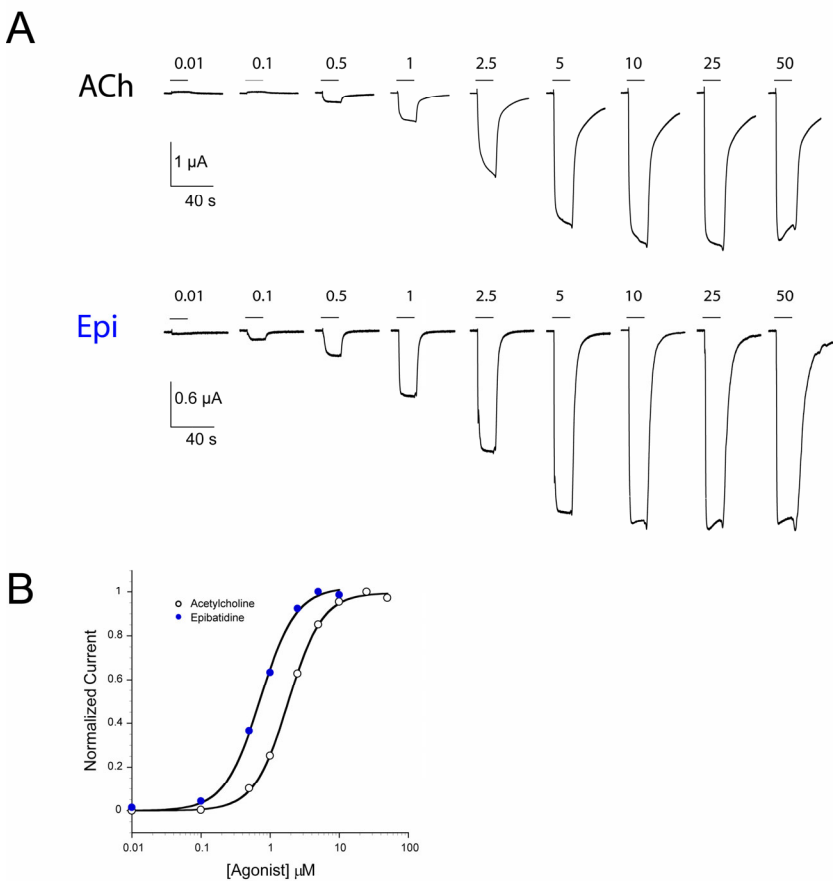

Figure 3.5. Electrophysiology Data. Electrophysiological analysis of ACh and epibatidine. **A)** Representative voltage clamp current traces for oocytes expressing nAChRs expressing the double mutant αD89NW149Wahβ$_9$'γδ. Bars represent application of ACh and epibatidine at the concentrations noted. **B)** Representative ACh (○) and epibatidine (●) dose-response relations and fits to the Hill equation for oocytes expressing αD89NW149Wahβ$_9$'γδ. Studies incorporate a βLeu9'Ser mutation.

3.3 DISCUSSION

The ability to understand drug-receptor interactions at nAChRs was dramatically improved with the appearance of the crystal structure of the ACh binding protein. It is important to remember that AChBP is not a functional ligand gated ion channel. Instead it is a soluble protein approximately 20 % homologous to the extracellular ligand-binding domain of nAChRs. Therefore, it is necessary to establish the relevance of interactions

predicted by the AChBP structures for functional nAChRs. The methodology of incorporating unnatural amino acids into these receptors provides a powerful functional tool to address this task.

A substantial network of hydrogen bonds with αD89 is implicated by various structural studies. The highly conserved collection of residues probed here seems well positioned to exert a strong influence on the structure and function of the nAChR. In the present work, we inserted a number of mutations, many quite subtle, into this region in order to probe the precise role of this proposed network. We examine the importance of the hydrogen bond network between the αD89 and backbone amides flanking αW149. Next, we probed the importance of the αD89 side chain on nAChR function. Finally, we aimed to understand elements that contribute to the diminished receptor function in αD89N mutant nAChR receptors. These studies demonstrate that αD89 provides structure for the agonist-binding site.

We discover that single backbone amides flanking αW149 are not critical hydrogen bond donors essential for normal nAChR function. Eliminating hydrogen bonds *1* and *3* through the αW149Wah mutation has a very modest effect on receptor function. Eliminating hydrogen bond *2* through the αT150Tah mutation has a larger effect, but still not overly large, especially when one considers that this mutation also attenuates the hydrogen bond between agonist and the backbone carbonyl of αW149 that is proposed to be important in binding nicotine and epibatidine. Earlier, Sine had shown that hydrogen bonds *4* and *5* are not critical to receptor function.[12] Therefore, elimination of one (or two) of the possible five hydrogen-bonding partners with αD89 is not sufficient to

dramatically reduce channel activity. It remains possible, however, that some combination of hydrogen bonds in this network is important for proper channel function.

In addition, this study probed the importance of the αD89 side chain on nAChR function. Mutations that altered side chain length and charge were incorporated at αD89: αD89E, αD89N, and αD89Nha. The αD89E mutation, introducing an extended side chain, resulted in a modest increase in ACh and epibatidine activity, and near wild-type activity for nicotine. Thus, the activity of nicotine tolerates the increased sterics of the αD89E side chain, while the activity of ACh and epibatidine is slightly impaired with this mutation. The αD89N mutation converts the negatively charged wild-type residue to a neutral residue at this position. The αD89N mutation also introduces an electrostatic clash between the amide side chain of αD89N and the backbone amide NHs surrounding αW149. Observations from the present work and previous studies by Sine and co-workers[12] revealed dramatically impaired nAChR receptors in the presence of an αD89N mutation. To further understand the impaired activity of the αD89N mutant, the present study addressed this issue in two ways.

First, we aimed to test the importance of negative charge in this region by incorporating nitro-homoalanine (Nha) at αD89 (αD89Nha) in the presence of ACh, nicotine, and epibatidine. Charge neutralization as achieved with the Nha residue has a only a moderate effect–an approximately 5-fold increase (≤0.9 kcal/mol) relative to the isosteric E residue for all agonists. Thus, it appears that although the charge at αD89 contributes favorably to receptor activity, it does not appear to be critical.

A second possibility for the diminished activity of receptors containing αD89N is that the amide group of the αD89N mutation introduces a detrimental electrostatic clash with

nearby backbone amides surrounding αW149. The αD89N mutation creates an electrostatic repulsion between the newly introduced $N^{\delta-}$–$H^{\delta+}$ bonds of the αD89N side chain and the backbone $N^{\delta-}$–$H^{\delta+}$ moieties at positions αW149 and αT150. The αW149Wah can almost completely rescue the αD89N mutation, apparently by removing the adverse $N^{\delta-}$–$H^{\delta+}$••• $N^{\delta-}$–$H^{\delta+}$ interaction. The αT150Tah mutation is partially successful in this regard. Therefore, we attribute the major destabilization caused by the αD89N mutation to this adverse electrostatic repulsion.

The positioning for αD89 with respect to the backbone appears to be slightly different for ACh and epibatidine than it is for nicotine. The agonist-binding site conformations of potent agonists ACh and epibatidine do not appear to tolerate disturbances near the αW149 backbone, while the nicotine-specific binding site conformation is able to accommodate some disturbances near this region. For example, an extended side chain at αD89 resulted in wild-type activity for nicotine, but resulted in a moderate decrease in activity for ACh and epibatidine. In addition, the flexibility of the nicotine-specific binding site enables relief of the electrostatic clash with either the αW149Wah or the αT150Tah mutant in the presence of αD89N. The more rigid binding sites for ACh and epibatidine are only able to relieve the electrostatic clash with the αD89N/αW149Wah mutant. It seems probable that potent agonists require optimal positioning of the backbone of αW149 to ensure proper alignment for a cation-π interaction with ACh and epibatidine. On the other hand, tight alignment does not appear necessary for nicotine, a weak agonist that does not utilize a strong cation-π interaction with αW149. Therefore, these chemical scale investigations provide insight into additional determinants that distinguish among the three agonists considered here.

It is important to note that the nitro group of Nha is also a much poorer hydrogen bond acceptor than the carboxylate of D/E, with estimates that a typical hydrogen bond would be reduced by 1-2 kcal/mol by such a substitution. The carboxylate of αD89 is proposed to be involved in four hydrogen bonds, yet the carboxylate to nitro conversion (E to Nha) is again destabilizing by ≤0.9 kcal/mol. Taken together, the results here suggest that the intricate network of hydrogen bonds implicated in several structures of the nAChR and AChBP is quite tolerant of modification. It may be that the functional significance of this region of the receptor is not as great as one might deduce from static, structural images alone.

In summary, a combination of unnatural amino acid mutagenesis and conventional mutagenesis has led to the following conclusions. We discovered that single backbone amides flanking αW149 are not critical hydrogen bond donors essential for normal nAChR function. It remains possible, however, that some combination of hydrogen bonds in this network is important for proper channel function. Structural requirements for the αD89 side chain were also elucidated. We determined that the negative charge at αD89 is moderately important for proper nAChR function. In addition, we determined that the electrostatic clash, introduced in the αD89N mutation, greatly contributes to the impaired function of this nAChR mutant. We also determined that the positioning of αD89 is more important for proper ACh and epibatidine function than for proper nicotine function.

3.4 Materials and Methods

Synthesis of Wah cyanomethyl ester (3-(3-Indolyl)-2-hydroxypropanoic acid cyanomethyl ester)

α-hydroxytryptophan (Wah) (3-(3-Indolyl)-2-hydroxypropanoic acid) cyanomethyl ester was synthesized according to previously published methods.[7,15] The hydroxy acid (255 mg, 1.24 mmol) was dissolved in 1.9 ml of $ClCH_2CN$ (30 mmol) and 514 µl Et_3N (3.65 mmol). Upon stirring under Ar for 45 min, the solution turned pale yellow. The reaction mixture was concentrated and dried under vacuum. The material was dry loaded onto a flash silica gel column and run in 9:1 methylene chloride/ethyl acetate to give 242 mg (80 % yield) of hydroxyl-tryptophan cyanomethyl ester: ^{1}H NMR (DMSO) δ 3.04 (m, 2H), 3.32 (broad s, 1H), 4.38 (broad s, 1H), 4.94 (s, 2H), 6.96 (t, 1H, J = 7.2 Hz), 7.05 (t, 1H, J= 6.9 Hz), 7.12 (d, 1H, J = 2 Hz), 7.32 (d, 1H, J = 7.8 Hz), 7.51 (d, 1H, J = 7.5 Hz) , 10.85 (s, 1H); ^{13}C NMR 30.0, 48.9, 70.7, 109.7, 111.3, 115.8, 118.3, 118.3, 120.8, 123.8, 127.3, 136.0, 172.7: Electrospray MS Calcd for $C_{13}H_{12}N_2O_3$ plus H: 245.08. Found *m/z* (M+H)*:* 245.0.

Synthesis of dCA-Wah

Hydroxy-tryptophan cyanomethyl ester (11 mg, 45 µmol) was dissolved in 315 µl dry DMF in a flame-dried 5 ml round bottom flask with a stir bar. The dinucleotide dCA (20 mg, 16.7 µmol) was added, and the reaction was stirred under Ar for 9 h. Upon completion of the reaction, the pure compound was obtained by preparative HPLC. Electrospray MS Calcd for $C_{30}H_{35}N_9O_{15}P_2$ minus H: 823.17. Found *m/z* (M-H): 822.0.

Synthesis of Nha-dCA

The synthesis of nitro-homoalanine was performed by Michael Torrice and is not included in the text of this chapter.

Electrophysiology

Stage VI oocytes of *Xenopus laevis* were employed. Oocyte recordings were made 24 to 48 h post injection in two-electrode voltage clamp mode using the OpusXpress^{TM} 6000A (Axon Instruments, Union City, California). Oocytes were superfused with Ca^{2+}-free ND96 solution at flow rates of 1 ml/min, 4 ml/min during drug application, and 3 ml/min during wash. Holding potentials were -60 mV. Data were sampled at 125 Hz and filtered at 50 Hz. Drug applications were 15 s in duration. Agonists were purchased from Sigma/Aldrich/RBI: (-) nicotine tartrate and acetylcholine chloride. Epibatidine was also purchased from Tocris as (±) epibatidine dihydrochloride. All drugs were prepared in sterile ddi water for dilution into calcium-free ND96. Dose-response data were obtained for a minimum of 10 concentrations of agonists and for a minimum of 3 cells. Dose-response relations were fitted to the Hill equation to determine EC_{50} and Hill coefficient values. EC_{50}s for individual oocytes were averaged to obtain the reported values.

Unnatural Amino Acid Suppression

Synthetic amino acids and α-hydroxy acids were conjugated to the dinucleotide dCA and ligated to truncated 74 nt tRNA as previously described.[15,16] Deprotection of amino acyl tRNA was carried out by photolysis immediately prior to co-injection with mRNA,

as described.[16, 17] Typically, 25 ng of tRNA were injected per oocyte along with mRNA in a total volume of 50 nl/cell. mRNA was prepared by *in vitro* runoff transcription using the Ambion (Austin, TX) T7 mMessage mMachine kit. Mutation to the *amber* stop codon at the site of interest was accomplished by standard means and was verified by sequencing through both strands. For nAChR suppression, a total of 4.0 ng of mRNA was injected in the subunit ratio of 10:1:1:1 of α:β:γ:δ. In all cases, the β subunit contained a Leu9'Ser mutation, as discussed below. Mouse muscle embryonic nAChR in the pAMV vector was used, as reported previously. In addition, the α subunits contain an HA epitope in the M3-M4 cytoplasmic loop for biochemical western blot studies. Control experiments show a negligible effect of this epitope on EC_{50}. As a negative control for suppression, truncated 74 nt or truncated tRNA ligated to dCA was co-injected with mRNA in the same manner as fully charged tRNA. At the positions studied here, no current was ever observed from these negative controls. The positive control for suppression involved wild-type recovery by co-injection with 74 nt tRNA ligated to dCA-Thr or dCA-Trp. Frame-shift suppression at αD89, performed by Michael Torrice, was utilized as described by Rodriguez et al.[18] The positive control for suppression involved wild-type recovery by co-injection with 74 nt tRNA(GGGU) ligated to dCA-Asp. In all cases, the dose-response data were indistinguishable from injection of wild-type mRNA alone.

Part B

Importance of αK145 in Channel Function

The AChBP structural data also revealed a potential role for Lys α145 (αK145), a highly conserved residue in the shell of amino acids immediately surrounding the agonist-binding box.[4,5] As shown in **Figure 3.6A**, the nicotine-bound AChBP structure places αK145 immediately adjacent to important aromatic binding site residues, Tyr α190 (αY190) and Tyr α93 (αY93). This agonist-bound structure reveals a possible interaction between the side chain of αK145 and the side chain of αY190.[5] Interestingly, this interaction is not present in the HEPES-bound AChBP structure, a cationic salt-bound structure assumed to be similar to the unbound state.[4] In this unbound structure, the αK145 side chain is 5.5 Å from the αY190 side chain.[4] This distance is decreased to 2.6 Å in the nicotine-bound structure.[5] Therefore, comparison of these two structures suggests that the αK145 side chain moves upon ligand binding to interact with the αY190 side chain (**Figure 3.6B**). It has been proposed that movement of this side chain could be involved in the nAChR channel-gating mechanism.[5] Because the AChBP is not a functional full-length channel, experimental studies are necessary to probe the functional significance of this proposed interaction on nAChR function.

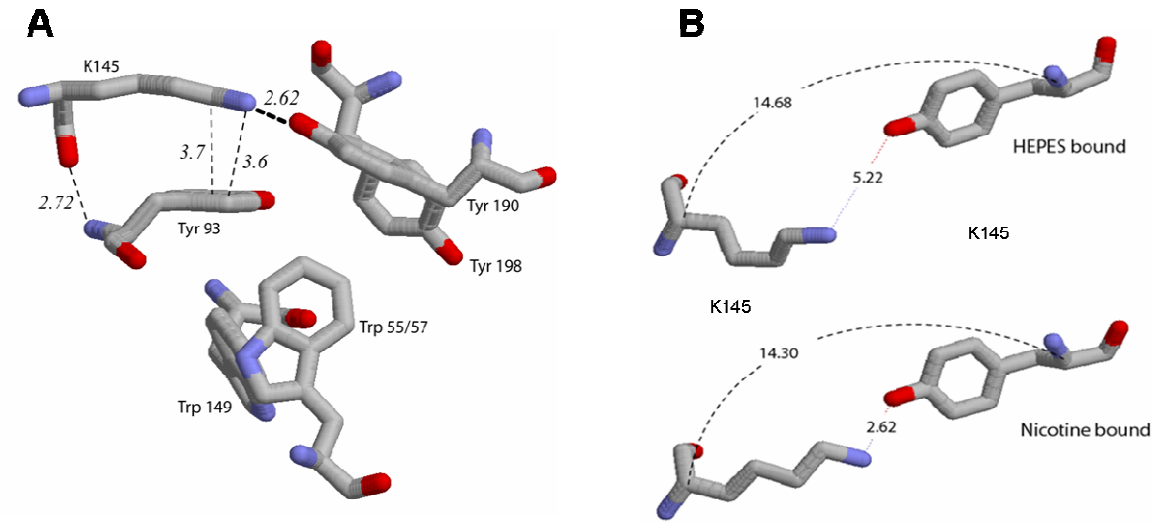

Figure 3.6. αK145 Interacts with Aromatic Binding Site Residues. A) The nicotine-bound AChBP structure positions αK145 adjacent to aromatic binding site residues. **B)** Comparisons of the agonist-bound and agonist free AChBP reveal side chain movement of αK145. αK145 backbone position remains relatively unchanged. Mouse muscle nAChR numbering is shown.

The present study aimed to evaluate the importance of the proposed hydrogen bond between αK145 and αY190 on nAChR channel function. To explore the importance of αK145, we incorporated conventional and unnatural amino acids at this position. The sequence alignment in **Figure 3.7** highlights the residues examined in this study. In particular we performed chemical scale investigations by utilizing *in vivo* nonsense suppression to incorporate an unnatural amino acid at αK145. This method offers a unique and powerful tool to subtly alter amino acid composition, a capability unavailable with conventional mutagenesis.

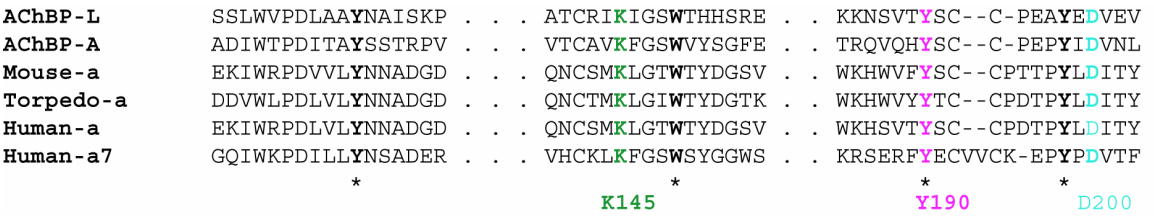

Figure 3.7. nAChR Sequence Alignment. The highly conserved residues αK145, αY190, and αD200 are highlighted. The four aromatic residues on the principal binding-site face are bolded and indicated with an asterisk.

Substitutions were incorporated at αK145 using both conventional and unnatural amino acid mutagenesis to examine the importance this side chain on nAChR function. Conventional side chain mutations that remove the positive charge or make the charge more diffuse were incorporated at αK145. In addition, an unnatural amino acid that shortens the Lys side chain was incorporated at αK145. These studies demonstrate the importance of the proper cationic character and length of the αK145 side chain for full nAChR function.

3.5 RESULTS

Unnatural amino acids were incorporated into the nAChR using *in vivo* nonsense suppression methods. The mutant receptors were evaluated using electrophysiology.[13] The structures of the conventional and unnatural amino acid substitutions utilized in this study are shown in **Figure 3.8**.

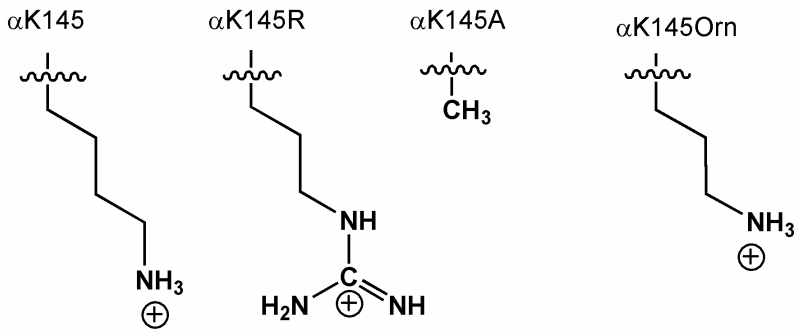

Figure 3.8. Amino Acid Side Chain Substitutions at αK145.

When studying weak agonists and/or receptors with diminished binding capability, it is necessary to introduce a Leu-to-Ser mutation at a site known as 9' in the second transmembrane region of the β subunit.[7,8] This 9' site in the β subunit is almost 50 Å from the binding site, and previous work has shown that a L9'S mutation lowers the effective concentration at half maximal response (EC_{50}) by a factor of roughly 40.[8,14] Results from earlier studies[8,14] and data reported below demonstrate that trends in EC_{50} values are not perturbed by L9'S mutations. In addition, the alpha subunits contain an HA epitope between M3-M4. Control experiments show a negligible effect of this epitope tag on EC_{50}.[7] It should be noted that the EC_{50} value is not a binding constant, but a composite of equilibria for both binding and gating.

Cationic Character of αK145 Important for Proper Channel Function

A comparison between the ligand-bound and the unbound AChBP crystal structures reveals a possible change in αK145 side chain position upon ligand binding. In the nicotine-bound structure, the αK145 side chain moves to a position with a favorable interacting distance with αY190 in comparison to the unbound-HEPES structure. To

probe the importance of the cationic head group at αK145, the side chain was mutated to a non-polar Ala residue, αK145A, and a more diffuse Arg residue, αK145R. The impact of these mutations on ACh EC_{50} is shown in **Table 3.4**. The more diffuse cationic side chain of the αK145R mutation resulted in an 8-fold *increase* in ACh EC_{50} and a 16-fold *increase* in epibatidine EC_{50} compared to wild-type receptors. The non-polar αK145A mutation resulted in a 27-fold *increase* in ACh EC_{50} and a 35-fold *increase* in epibatidine EC_{50} compared to wild-type nAChRs. These studies demonstrate the importance of a localized positive charge of the αK145 side chain on ACh and epibatidine activity.

Attempts to record nicotine dose-response relations for αK145A and αK145R were unsuccessful. It is possible that EC_{50} measurements require nicotine doses that would block the nAChR αK145R. In addition a low efficacy of nicotine (<5 %) was observed compared to ACh. Therefore, nicotine EC_{50} values are not reported for these mutants.

Table 3.4 Probing αK145 Side Chain [a]

Agonist	αK145[b]	αK145R	αK145A	αK145Orn
ACh	0.83 ± 0.04	6.6 ± 0.6	22 ± 2	41 ± 4
Epi	0.60 ± 0.04	9.5 ± 0.9	21 ± 4	-- [c]

[a] EC_{50} (μM) ± standard error of the mean. The receptor has a Leu9'Ser mutation in M2 of the β subunit. [b] Data reported previously.[7] [c] Value difficult to obtain due to insufficient signal.

Side Chain Length of αK145 Important for Proper Channel Function

To probe the importance of the interaction between αK145 and αY190 for ACh activity, a chemical-scale analysis was performed using *in vivo* nonsense suppression. A Lys analogue, ornithine (Orn), containing the same cationic head group as a Lys but a

side chain shortened by one carbon, was incorporated at αK145 (αK145). Therefore, the side chain length of αK145Orn may not be sufficient to make the proposed hydrogen bond with αY190 upon agonist binding. The impact of this shortened side chain mutation on ACh activity is shown in **Table 3.4**. The αK145Orn mutation resulted in a dramatic 49-fold *increase* in ACh EC_{50} compared to wild-type nAChRs. These studies demonstrate the significance of αK145 side chain length on ACh activity.

It is important to note that channel expression was very difficult to measure for the αK145Orn mutation. To obtain sufficient signal to monitor protein function in the presence of ACh, the incubation time of the oocyte was increased to 5 or 6 days post injection. Control experiments, performed on ACh wild-type recovery of αK145K using *in vivo* nonsense suppression under similar conditions, reveal dose-response data that were indistinguishable from those resulting from injection of wild-type mRNA alone. Studies of the αK145Orn mutant in the presence of epibatidine and nicotine were unable to generate an EC_{50} value due to the poor expression of this mutant and to the lower efficacy of epibatidine compared to ACh. Therefore, an epibatidine EC_{50} value is not reported for this mutant.

3.6 DISCUSSION

The ability to understand drug-receptor interactions at nAChRs was dramatically improved with the appearance of the crystal structure of the ACh-binding protein. It is important to remember that AChBP is not a functional ligand gated ion channel. Instead it is a soluble protein approximately 20 % homologous to the extracellular ligand-binding domain of nAChRs. Therefore, it is important to establish the relevance of interactions

predicted by the AChBP structures for functional nAChRs. In addition, these structures are only a static picture of the protein and do not provide information on how these dynamic proteins transition from one conformation to another. Thus, more knowledge of protein transitions on the atomic level is still needed to fully understand the gating mechanisms of these membrane proteins. The methodology of incorporating unnatural amino acids into these receptors provides a powerful functional tool to address these questions.

In the present work, we probed the importance of the αK145 side chain on ACh and epibatidine activity. We examined the importance of a localized cationic charge on the αK145 side chain. In addition we examined the importance of αK145 side chain length on ACh activity. We concluded that cationic head group character at αK145 must be preserved for proper ACh and epibatidine activity. In addition, we concluded that side chain length is important for ACh activity. These data support observations from the AChBP ligand-bound and ligand-free structures that propose the formation of a hydrogen bond between αK145 and binding-site residue αY190 upon ligand binding.

During the progress of our studies, Sine and co-workers published results consistent with our findings.[19] Single-channel kinetic analyses were performed on αK145 mutations to probe the importance of the αK145/αY190 interaction. Sine and co-workers also examined the role of a nearby Asp residue, αD200 in stabilizing αK145 in the unbound nAChR receptor (**Figure 3.9**). The removal of the cationic side chain with the Ala mutation at αK145 (αK145A) and the more subtle change of a neutral side chain with similar shape, a Gln mutation at αK145 (αK145Q), were studied. Both mutations were found to significantly impair channel gating. A kinetic analysis of the single-

channel data of the αK145A and αK145Q mutants revealed a role for αK145 in the nAChR gating process. Similar observations were made for the αD200N single mutant and the αK145Q/αD200N double mutant. Sine and co-workers also examined the impact of αY190 on channel function. Not surprisingly, mutations at this aromatic binding-site residue were not tolerated, an observation consistent with previous studies.[14] Researchers concluded that αD200 and αK145 are interdependent residues involved in coupling agonist binding to channel gating.

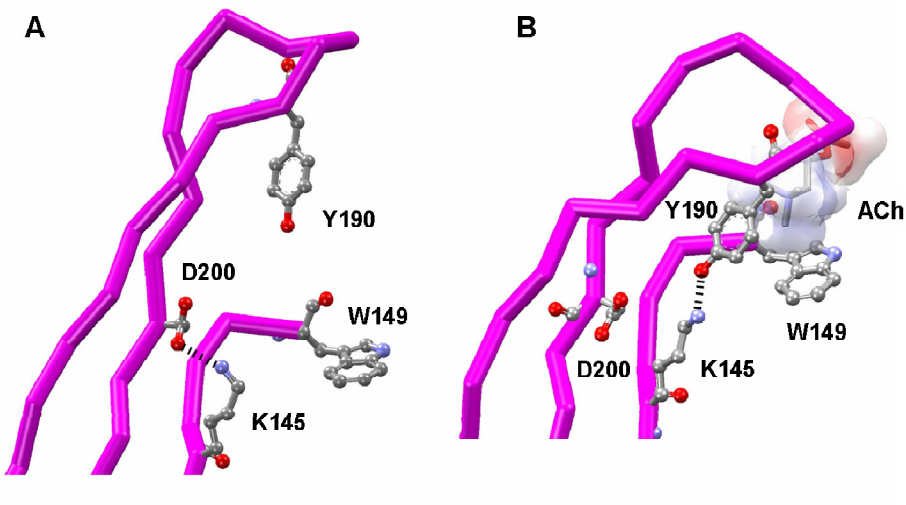

Figure 3.9. **Movement of αK145 in Agonist-Free and Agonist-bound AChBP**.[19] **A**) Agonist-free structure generated by Sine and co-workers from HEPES-bound AChBP.[19] αD200 interacts with αK145 in this unbound state. **B**) ACh-bound AChBP structure generated from docking studies by Sine and co-workers from HEPES-bound AChBP. The interaction between αD200 and αK145 is transferred to αK145 and αY190 in the agonist-bound structure. Mouse muscle nAChR numbering indicated. Figure reproduced from reference 19 with copyright permission of The Rockefeller University Press.

The observed changes in channel gating kinetics with the αK145 and αD200 mutations revealed that these residues are important in channel opening, leading Sine and co-workers to conclude that this network of residues relays the initial information from ligand binding to channel gating.[19] These studies can be expanded to identify additional

residues that couple agonist binding to channel gating. Data generated from the present study are consistent with these observations and further confirm the importance of the highly conserved αK145 residue for proper channel function.

3.7 MATERIALS AND METHODS

Synthesis of $(NVOC)_2$Ornithine ((S)-2,5-bis((4,5-dimethoxy-2-nitrobenzyloxy) carbonylamino)pentanoic acid)

L-Ornithine-hydrochloride (Advanced Chem Tech Y02595) (90 mg, 0.53 mmol) was added to 1.2 ml of 10 % Na_2CO_3 (0.53 mmol) and 1.8 ml dioxane. The reaction was stirred over an ice bath and 4,5 dimethoxy-2-nitrobenzyl chloroformate (NVOC-Cl, Aldrich) (453 mg, 1.6 mmol) was slowly added to the mixture. The reaction was allowed to warm to ambient temperature. After 4 h, the reaction was poured into 30 ml of water and extracted 3 times with 20 ml of diethyl ether. The precipitate was filtered to give 475.4 mg of crude $(NVOC)_2$–Ornithine (45.8 % crude yield): Electrospray MS Calcd for $C_{25}H_{30}N_4O_{14}$ minus H: 609.18. Found *m/z* (M-H)*:* 609.2.

Synthesis of $(NVOC)_2$Ornithine cyanomethyl ester ((S)-cyanomethyl 2,5-bis((4,5-dimethoxy-2-nitrobenzyloxy)carbonylamino)pentanoate

The crude (NVOC)2-Ornithine (250 mg, 0.41 mmol) was dissolved in 1 ml of $ClCH_2CN$ (15.8 mmol) and 200 µl Et_3N (1.4 mmol). The reaction was stirred under Ar for 50 min and was concentrated and dried under vacuum. The material was purified on a flash silica gel column and run in 3:1 methylene chloride/ethyl acetate to give 130 mg (49 % yield) of $(NVOC)_2$Ornithine cyanomethyl ester: ^{1}H NMR ($CDCl_3$) δ 1.64 (m, 2

H), 1.76 (m, 2H), 3.25 (m, 2H), 3.96 (t, 12H, J = 7 Hz), 4.44 (m, 1H), 4.77 (m, 2H), 4.99 (broad s, 1H, **αNH**), 5.52 (m, 4H), 6.98 (d, 2H, J = 5 Hz), 7.67 (d, 2H, J = 5Hz); ^{13}C NMR 26.27, 40.32, 49.12, 53.60, 56.44, 56.51, 63.86, 64.26, 108.25, 110.16, 110.71, 113.84, 127.52, 139.75, 140.05, 148.29, 153.47, 153.70, 155.56, 156.15, 170.96: Electrospray MS Calcd for $C_{27}H_{31}N_5O_{14}$ plus Na: 672.18. Found *m/z* (M+ Na+)*:* 672.2.

Synthesis of dCA-(NVOC)$_2$Ornithine

(NVOC)$_2$Ornithine cyanomethyl ester (25 mg, 45 μmol) was dissolved in 315 μl dry DMF in a flame-dried 5 ml round bottom flask with a stir bar. The dinucleotide dCA (20 mg, 16.7 μmol) was added, and the reaction was stirred under Ar for 2 h. Upon completion of the reaction, the pure compound was obtained by preparative HPLC. Maldi TOF MS Calcd for $C_{44}H_{53}N_{12}O_{26}P_2$ plus H: 1228.26. Found *m/z* (M+H): 1229.4.

Electrophysiology

Stage VI oocytes of *Xenopus laevis* were employed. Oocyte recordings were made 24 to 48 h post injection in two-electrode voltage clamp mode using the OpusXpress^{TM} 6000A (Axon Instruments, Union City, California). Extended incubation periods of 72 to 96 h were required for the αK145Orn studies. Oocytes were superfused with Ca^{2+}-free ND96 solution at flow rates of 1 ml/min, 4 ml/min during drug application, and 3 ml/min during wash. Holding potentials were -60 mV. Data were sampled at 125 Hz and filtered at 50 Hz. Drug applications were 15 s in duration. Agonists were purchased from Sigma/Aldrich/RBI: (-) nicotine tartrate and acetylcholine chloride. Epibatidine was purchased from Tocris as (±) epibatidine. All drugs were prepared in sterile ddi water for

dilution into calcium-free ND96. Dose-response data were obtained for a minimum of 10 concentrations of agonists and for a minimum of 4 cells. Dose-response relations were fitted to the Hill equation to determine EC_{50} and Hill coefficient. EC_{50}s for individual oocytes were averaged to obtain the reported values.

Unnatural Amino Acid Suppression

Synthetic amino acids and α-hydroxy acids were conjugated to the dinucleotide dCA and ligated to truncated 74 nt tRNA as previously described.[15,16] Deprotection of aminoacyl tRNA was carried out by photolysis immediately prior to co-injection with mRNA, as described.[16, 17] Typically, 25 ng of tRNA were injected per oocyte along with mRNA in a total volume of 50 nl/cell. mRNA was prepared by *in vitro* runoff transcription using the Ambion (Austin, TX) T7 mMessage mMachine kit. Mutation to the *amber* stop codon at the site of interest was accomplished by standard means and was verified by sequencing through both strands. For nAChR suppression, a total of 4.0 ng of mRNA was injected in the subunit ratio of 10:1:1:1 for α:β:γ:δ. In all cases, the β subunit contained a Leu9'Ser mutation, as discussed below. Mouse muscle embryonic nAChR in the pAMV vector was used, as reported previously. In addition, the α subunits contain an HA epitope in the M3-M4 cytoplasmic loop. Control experiments show a negligible effect of this epitope tag on EC_{50}.[7] The positive control for suppression involved wild-type recovery by co-injection with 74 nt tRNA ligated to dCA-Lys. In all cases, the dose-response data were indistinguishable from injection of wild-type mRNA alone.

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