Chapter 3

Investigations of pH dependence of Loop 2 of the nAChR α_1 subunit

3.1 Introduction

Cys-loop ligand gated ion channels mediate rapid synaptic transmission in the central and peripheral nervous systems. Receptors for acetylcholine (nicotinic acetylcholine, nACh) and serotonin (5-HT_3) are classified excitatory (cation conducting) while receptors for γ-aminobutyric acid (GABA) and glycine (Gly) are termed inhibitory (anion conducting). Proper functioning of these receptors is crucial to normal brain function. As such, malfunctions in these receptors lead to a number of "channelopathies" making the receptors a target of pharmaceutical efforts for a range of neurological disorders, including addiction, Parkinson's disease, Alzheimer's disease, schizophrenia, and depression.[1,2]

At rest Cys-loop receptors are in a closed non conducting state. Upon binding of their cognate neurotransmitter (agonist), the channels undergo a conformational change to an open ion-conducting state. The conformational change is analogous to the opening of a gate, and the process of going from a closed channel to an open one is often referred to as channel gating. The gating mechanism for this superfamily of receptors is one of the most challenging questions in molecular neuroscience. The neurotransmitter binding site is located 50-60 Å from the channel gate, and it remains unclear how binding of a small organic molecule can induce a structural change in a large, multisubunit, integral membrane protein.

The superfamily shares a common topology of five homologous subunits arranged pseudo-symmetrically around a central ion-conducting pore. Each subunit (Figure 3.1) contains a large amino-terminal extraceullular domain comprised of two beta sheets, followed by four membrane-spanning helices (M1-M4) and a short extracellular carboxy tail. The M2 helix of each subunit lines the channel pore and contains the channel gate. Ligand binding sites are located in the extracellular domain at select subunit interfaces. Numerous biochemical studies have established the importance of loops 2 and 7 (Figure 3.1 C, D) in the extraceullular domain and the M2-M3 linker as critical to channel gating.[3-9]

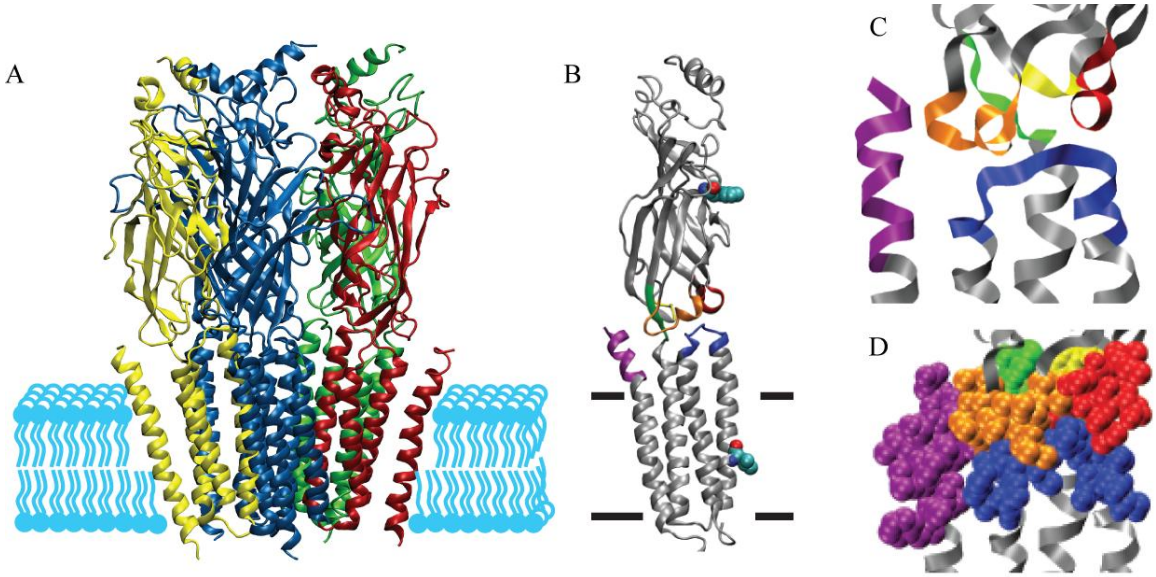

Figure 3.1 Cys-loop ligand gated ion channel structure. (**A**) The *Torpedo* nAChR from PDB 2BG9 is nearly identical to the mouse muscle nAChR. α subunits are shown in blue, β in green, δ in red, and γ in yellow. A single subunit (**B**) with the ligand binding site and channel gate highlighted as Van der Waals residues. A geometrically defined gating interface (**C**) in ribbon and space fill models (**D**) from the 2BG9 structure. Loop 2 is shown in red, loop 7 in orange, and the M2-M3 linker in blue.

As discussed in Chapter 2,[10] studies in the mouse muscle nAChR have demonstrated the important balance of charged residues at the gating interface of Cys-loop receptors. Loop 2 was of particular interest to us as introduction of positive charge

at four consecutive residues (D44, E45, V46, and N47) in this loop lowered EC_{50}.[3,10] In the nAChR α_1 subunit the first two residues of Loop 2 are aspartate and glutamate (positions 44 and 45, respectively). D44 is conserved among the nACh α subunit and E45 is highly conserved as a negatively charged residue among the superfamily. The short length of loop 2 and high degree of conservation of charge (Table 2.1) suggested there may be a perturbed pK_a in this region of the protein. Therefore, we hypothesized that the region may be sensitive to changes in pH and that this sensitivity results from changes in the protonation state of the side chains.

3.2 Results

On average the pK_a of the side chain carboxylic acid of aspartate and glutamate in a protein are 3.65 and 4.25, respectively,[11] and thus the residues are in the carboxylate form at physiological pH. If, however, loop 2 has an altered pK_a due to the number of charged species, it is possible that one of the D44 and E45 side chains is protonated. To address this possibility, we both lowered and raised the pH of the extracellular recording solution, normally a pH of 7.5, by two pH units (pH=5.5 and 9.5, respectively). The change in pH did not significantly impact EC_{50} (Table 3.1) indicating that the receptor is not highly sensitive to pH and that a perturbed pK_a at loop 2 is unlikely.

Table 3.1 EC_{50} (μM) of loop 2 histidine mutations grouped by pH

	pH = 5.5		**pH=7.5**		**pH=9.5**	
	EC_{50}	n_H	EC_{50}	n_H	EC_{50}	n_H
Wild Type	64 ± 2	1.4 ± 0.1	50 ± 2	1.6 ± 0.1	71 ± 1	1.5 ± 0.1
D44H	45 ± 1	1.5 ± 0.1	41 ± 2	1.6 ± 0.1	48 ± 1	1.5 ± 0.1
E45H	4.3 ± 0.1	1.5 ± 0.1	5.8 ± 0.3	1.4 ± 0.1	5.3 ± 0.2	1.5 ± 0.1
V46H	75 ± 0.2	1.4 ± 0.1	61 ± 3	1.5 ± 0.1	94 ± 6	1.3 ± 0.1

Ideally, the pH would be lowered even further to modulate the protonation state of the D44 and E45 side chains. However, the health of the oocytes degrades at pH < 5.5. Thus we needed to incorporate a side chain that would change from neutral to positively charged when the pH was lowered to 5.5 from 7.5. In Chapter 2,[10] we found that introduction of a positive charge at D44, E45, and V46 decreased EC_{50}, thus these residues were selected for further study. At physiological pH, the imidazole side chain of histidine is neutral (Figure 3.2), but at pH<6.0 the imidazole ring is charged. By first incorporating histidne at the site of interest, we would thus be able to alter the protonation state, and thus the charge of the side chain by changing the pH of the extracellular media.

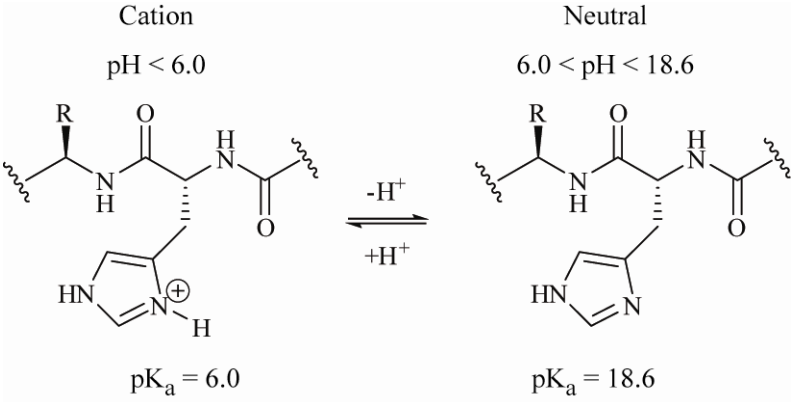

Figure 3.2 Protonation states of a histidine residue. At physiological pH, the imidazole side chain is neutral.

The EC_{50} of D44H was essentially that of wild type at physiological pH. Neither decreasing nor increasing the pH altered this EC_{50} (Table 3.1). In contrast, incorporation of histidine at position 45, greatly impacted channel function, lowering the EC_{50} ~10 fold. Lowering the pH had a small affect on EC_{50} while raising the pH did not. At the third postion, V46, mutation to histidine gave an EC_{50} similar to wild type. Both lowering and raising the pH increased EC_{50} slightly (1.5-fold and 2-fold, respectively) similar to wild type. The shape of the dose-response relationships for wild type and the mutant receptors

remains unchanged with varying pH (Figure 3.3), indicating the receptor is able to function properly.

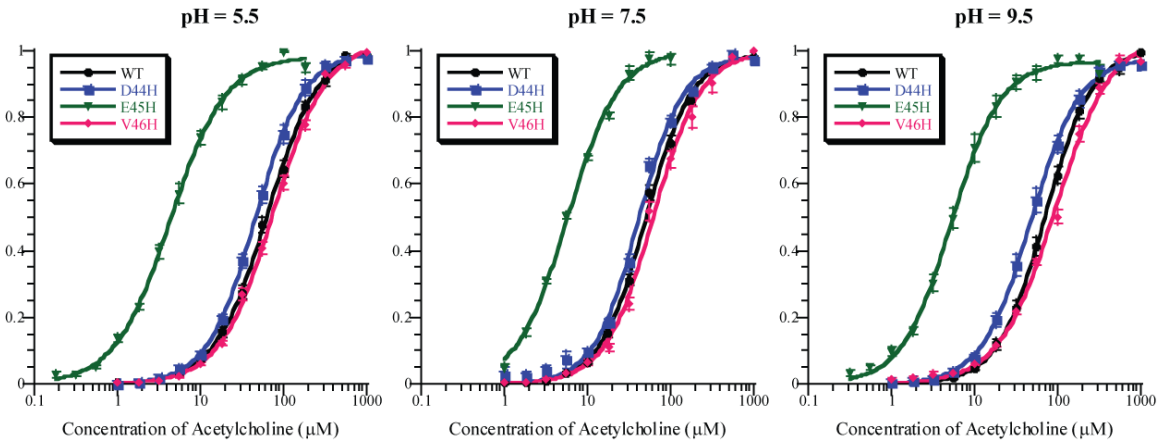

Figure 3.3 Dose response relationship for wild type (black), D44H (blue), E45H (green), and V46H (pink) mutants at varying pH. The y-axis corresponds to the whole cell current of each cell after normalizing to the maximal current for the given cell.

3.3 Discussion

Shifts in the EC_{50} of the wild type muscle nAChR do not show a one-directional trend with pH (Table 3.1). Rather, the EC_{50} increases with both an increase and decrease in pH. These changes in EC_{50} could be intrinsic to the receptor or the result of changes in the concentration of acetylcholine due to hydrolysis. Increasing the time between making acetylcholine solutions and conducting electrophysiology recordings resulted in higher EC_{50} values, suggesting degradation of acetylcholine contributes to the changes in EC_{50}. Ester hydrolysis of acetylcholine produces choline, a much weaker agonist of the receptor. Drug solutions were prepared by identical means for both the wild type and mutant receptors. We use the changes in EC_{50} for wild type, which accounts for both the intrinsic response of the protein to pH and degradation of acetylcholine as a reference point for the mutant receptors. The ratio of the EC_{50} at low pH to that of the

physiological pH is denoted as $R_{pH,5.5}$ and has a value of 1.28±0.06 for wild type. For the higher pH, the wild type receptor has an $R_{pH,9.5}$ equal to 1.42±0.06.

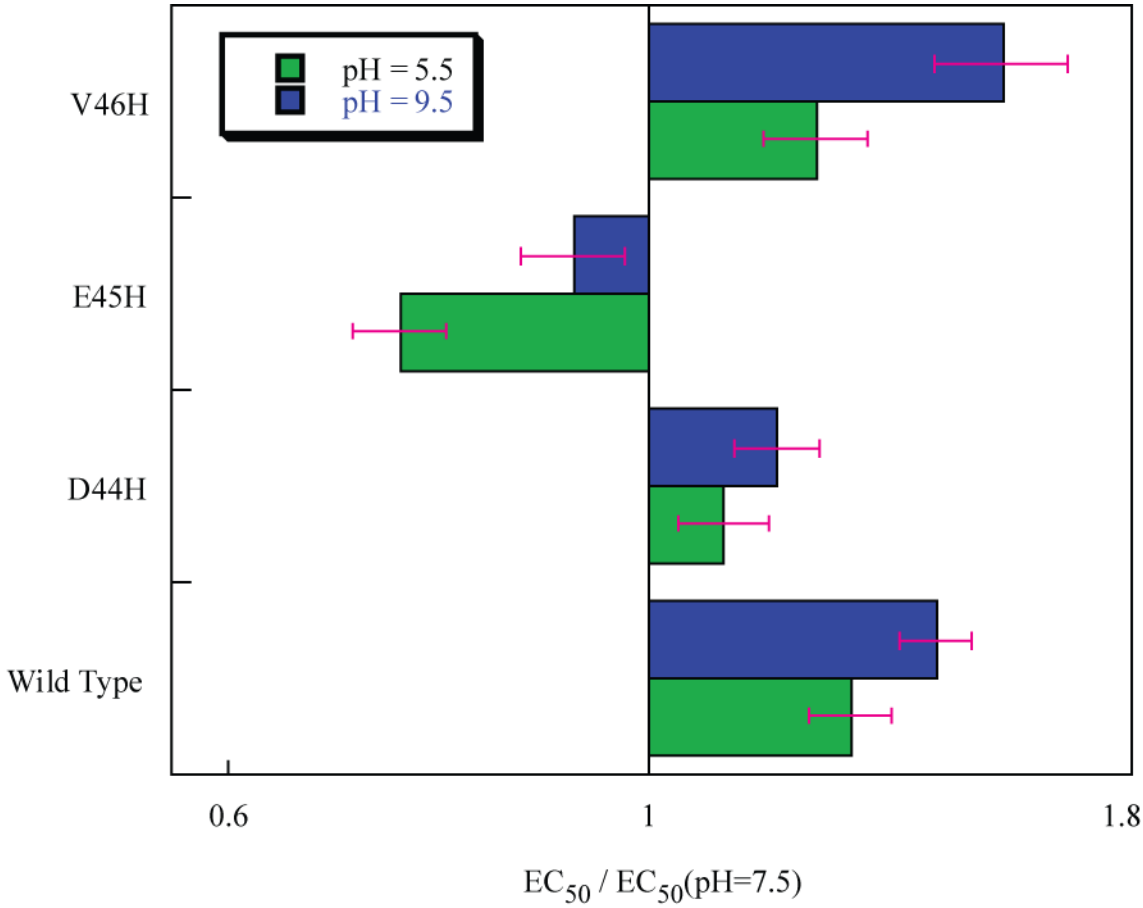

Figure 3.4 Shift in EC_{50} due to pH. Physiological pH (7.5) is used as the reference point. E45H, and to a lesser extent D44H, differ from wild type. A log scale is used for the x-axis.

In all cases the changes in EC_{50} due to altering the pH are small, but not meaningless. Of the three mutations, V46H is most similar to wild type while E45H differs to the greatest extent. The pH range used in these experiments is sufficient to form the positively charged histidine species (Figure 3.2), therefore we will focus our further discussion to the low pH experiments. Previous studies,[10] demonstrate that neutralizing the charge by conventional mutation to D44N or E45Q lowers EC_{50} and that

the affect is greater at E45. These data are consistent with the EC_{50} values obtained for D44H and E45H at physiological pH. While the EC_{50} for D44H is essentially that of wild type, we see a l0-fold decrease in EC_{50} for E45H.

Based on the consistency of these results with other studies,[10] we expected to see a decrease in EC_{50} for all three histidine mutants when the pH was lowered, creating a bias for the positively charged histidine side chain. Only E45H shows the expected decrease in EC_{50} and the change is quite small. However, we must also account for the slight increase in EC_{50} from hydrolysis of acetylcholine as seen for wild type. To complete this analysis, it is best to take the ratio of R_{pH} for the mutant ($R_{pH,mutant}$ = $EC_{50,mutant}/EC_{50(pH=7.5),\ mutant}$) to R_{pH} for wild type to give a ratio of ratios (Figure 3.5). Once the acetylcholine hydrolysis is accounted for, the data clearly indicate that lowering the pH (Figure 3.5, green bars) has a similar affect on the E45H channel function as incorporating a positively charged residue at this site.[10] D44H follows the trend of E45H though the effects are smaller.

The results for V46H are virtually identical to that of the wild type receptor (Figure 3.5, top). Although the mutation V46K drastically lowers EC_{50}[10] indicating a positively charged species increases channel function, we were not able to affect this same result using an altered pH in the extracellular media. Our V46H data are more indicative of the V46R results (2-fold increase in EC_{50}) reported in the same study.[10] Unlike the side chains of D44 and E45 which are suggested to stick into open space in the available structural data,[12] it has been proposed that the V46 side chain is in a hydrophobic environment.[12-16] Our results are consistent with this proposal. If the V46H

side chain is in a hydrophobic environment, it would not be readily in contact with the extracellular solution and therefore would not be affected by changes in pH.

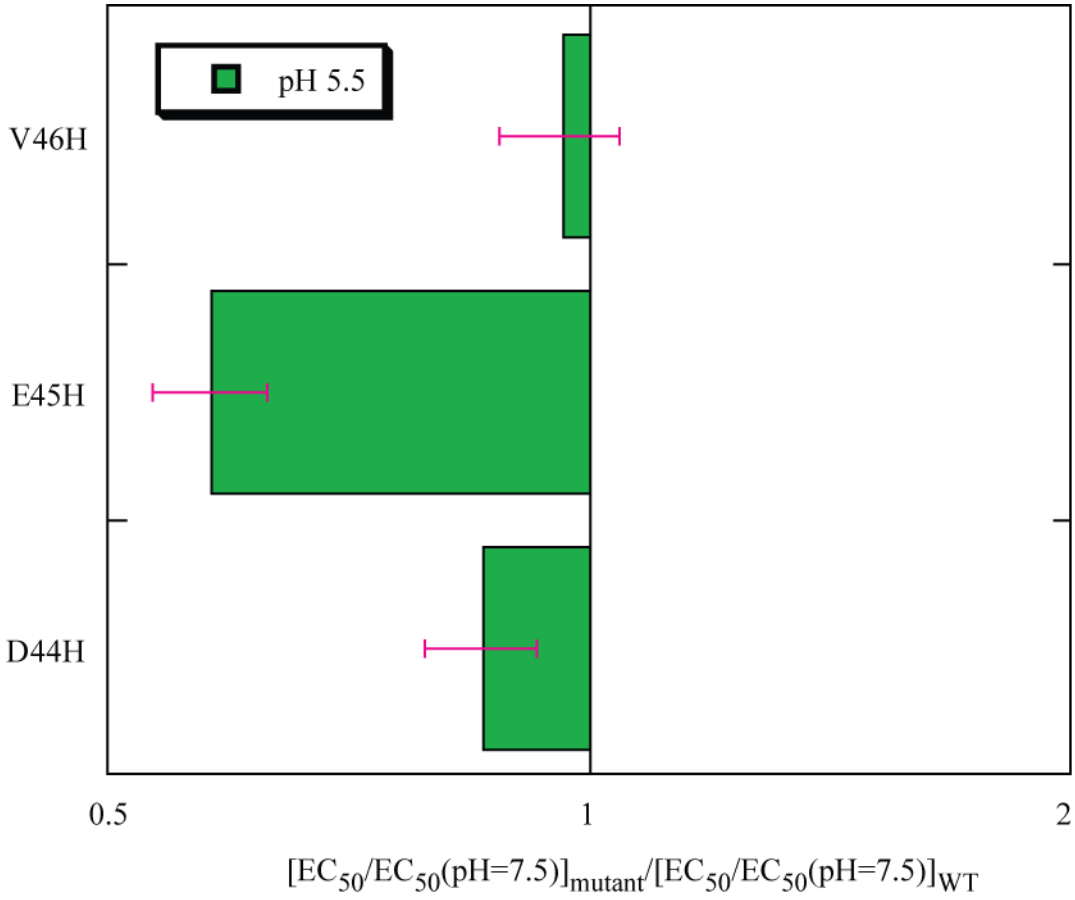

Figure 3.5 Comparison of shifts in EC_{50} from pH compared to the same shift found in wild type. Wild type is set to unity (center of graph). A log scale is used for the x-axis.

The muscle type nAChR does not show a pH dependence, indicating that despite being short and highly charged, the loop 2 region of the α_1 subunit does not experience a perturbed pK_a. Additionally, we have found that the affects of charge reversal[10] at two loop 2 residues, D44 and E45, can be mimicked by mutating these residues to histidine and varying the pH. At a third loop 2 resiude, V46, we find no alteration in channel function due to changes in pH. While these experiments do not definitively place V46 in

a hydrophobic environment, [12-16] the results suggest that unlike D44 and E45, V46H is in a sequestered environment, unable to interact with the extracellular solution.

3.4 Materials and Methods

Mutagenesis and mRNA Synthesis: Mutations in the α subunit were made following the QuickChange mutagenesis protocol (Strategene). Custom primers were purchased from Integrated DNA technologies. The mRNA that codes for the muscle type nAChR subunits (α, β, δ, and γ) was obtained by linearization of the expression vector (pAMV) with NotI (Roche), followed by *in vitro* transcription using the mMessage mMachine kit purchased from Ambion (Austin, TX). RNA concentrations were determined by UV/Vis spectroscopy.

Electrophysiology and Data Analysis: mRNAs of α, β, δ, and γ subunits were mixed in the ratio of 2:1:1:1 and microinjected into stage VI oocytes of *Xenopus laevis*. The mRNA mixes were diluted to a final concentration of 10 ng/μl. Oocytes were injected with 50 nL mRNA mix each, then stored in $ND96^{+}$ with 5% horse serum at 16-18°C. Oocytes were shaken continuously during incubation. Electrophysiology recordings were performed 24–48 hours after injection in two-electrode voltage clamp mode using the OpusXpress 6000A (Axon Instruments, Molecular Devices). The holding potential was -60 mV and agonist was applied for 15 seconds,[17] with 146 seconds of wash time between doses of agonist. For all dose-response data, agonist was applied from lowest to highest concentration. Dose-response data were obtained for at least eight concentrations of agonists and for a minimum of five oocytes with I_{max} equal to or greater than 500 nA. EC_{50} and Hill coefficients (n) were calculated by fitting the dose-response

relation to the Hill equation (Equation 3.1). All data are reported as mean ± standard error of the fit.

$$I = \frac{I_{max}}{1 + EC_{50}/[A]^n}$$ **Equation 3.1**

Acetylcholine chloride was purchased from Sigma. Stock solutions of 1M acetylcholine in water were stored at -20°C. Drug solutions were made from dilution of 1M acetylcholine using calcium-free ND96 buffer.

Drug Solutions for pH=5.5, 9.5: To prevent unnecessary hydrolysis of the acetyl group of acetylcholine, stock solutions of acetylcholine were made up in calcium free ND96 pH=7.5. No more than one hour prior to recording, the stock solutions were diluted 1- to 100-fold using calcium free ND96, previously pHed to 5.5 (using concentrated hydrocholoric acid) or 9.5 (using concentrated sodium hydroxide). This process was used for each individual experiment on the OpusXpress.

3.4 References

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