Chapter 1 Introduction to the Thesis

The nicotinic acetylcholine receptor (nAChR) is one member of the group of transmembrane receptors known as ligand gated ion channels, which are opened upon binding of ligand to the extracellular surface. The three main superfamilies of these receptors are the nicotinic acetylcholine receptor family (for review, see Changeux and Edelstein (1998)), the glutamate receptors (GluR) (reviewed in Paas (1998)), and the P2X receptors (reviewed in Buell *et al.* (1996)). The nAChR family is composed of channels gated by the neurotransmitters acetylcholine (ACh), GABA, glycine, and serotonin ($5HT_3$). Although the nAChR and $5HT_3$ receptors conduct cations, while $GABA_A$ and glycine receptors conduct anions, there is still ~25%-60% sequence homology between their subunits. Within the GluR family, with 18-70% sequence identity, all of the channels are gated by glutamate but are further subdivided based on sensitivity to other drugs, AMPA, kainate, and NMDA. The P2X receptors, which have 36-48% sequence identity between subunits, are all gated by ATP.

The proposed topology of these channels was elucidated first by hydropathy plots and further by biochemical and mutational analyses (Figure 1-1). The subunits of the nAChR family have four transmembrane spanning segments, with extracellular N- and C-termini, and the channel is formed by a pentamer of subunits (Karlin and Akabas, 1995). Originally, the GluR subunits were proposed to have four transmembrane segments by analogy to the nAChR. However, subsequent data indicates that the subunits consist of 3 transmembrane spanning segments, with extracellular N-termini and intracellular C-termini. The N-terminal segment and residues between the second and third transmembrane segment contribute to the agonist binding site (Paas, 1998). A hairpin loop between the first and second transmembrane segments contributes to the channel but does not cross the bilayer, similar to K^+ channels (Kuner *et al.*, 1996). Additionally, like

K^+ channels, the GluR family forms tetramers (Laube *et al.*, 1998; Mano and Teichberg, 1998). The trimeric P2X receptors (Nicke *et al.*, 1998) are composed of subunits with only 2 transmembrane segments, with the N- and C-termini on the intracellular side of the membrane (Newbolt *et al.*, 1998; Rassendren *et al.*, 1997).

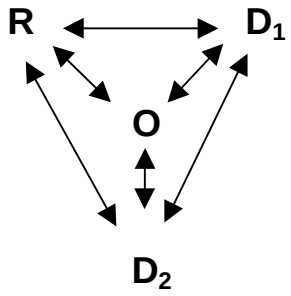
Multiple receptor isoforms can be generated from the variety of homologous subunits available. The composition of subunits of the nAChR varies in the two subtypes, muscle-type and neuronal. In *Torpedo* and embryonic muscle nAChR, the nAChR is composed of $\alpha_2\beta\gamma\delta$, while in adult muscle the subunit composition is $\alpha_2\beta\varepsilon\delta$. Neuronal nAChR can consist of either α homomultimers or combinations of α and β (non-α) subunits, with 8 known neuronal α subunits and 3 known neuronal β subunits (Role and Berg, 1996). Within the brain the main receptor isoforms contain either $\alpha4$ and $\beta2$ or $\alpha2$ and $\beta4$ subunits, though the ratio of subunits which form the pentamer is unknown. Six α, 3 β, 3 γ, 1 δ, 1 ε, 1 π, and 3 ρ subunits of the $GABA_AR$ have been identified (Mehta and Ticku, 1999). The $GABA_AR$ is the primary inhibitory channel of the central nervous system, and the major adult isoform is $\alpha1\beta2\gamma2$ (McKernan and Whiting, 1996), with a proposed stoichiometry of $\alpha1_2\beta2_2\gamma2$ (Chang *et al.*, 1996). The glycine receptor, the main inhibitory channel of the spinal cord, has 3 α and 1 β subunits with which to form channels (Rajendra *et al.*, 1997).

The glutamate receptors, the primary excitatory channels of the brain, can be formed by several subunits. The AMPA receptors are composed of the GluR1-4 subunits, while the KA receptors are composed of the GluR5-7, KA1, and KA2 subunits. The NMDA receptors have two types of subunits, NR1 and NR2A-D (Barnard, 1997). The 7 homologous P2X (Chang *et al.*, 1996) subunits are expressed throughout the nervous system, as well as in immune cells, glands, and muscle (Buell *et al.*, 1996).

The most studied ligand-gated ion channel is the nAChR. This focus is due to the relative ease with which large amounts of the nAChR can be isolated. The *Torpedo* ray fish uses acetylcholine as the neurotransmitter for stimulation of its electric organs. The postsynaptic membrane fragments can easily be isolated from the organ, yielding nAChR as ~50% of the isolated protein. From each fish, ~100 nmoles of nAChR can be isolated from this simple preparation.

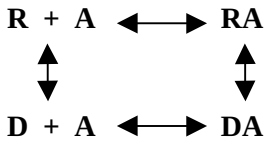
The amount of nAChR that can be isolated has even allowed structural imaging studies (Figure 1-2) (Unwin, 1998). When the isolated post-synaptic membranes are incubated at room temperature, they form a 2-D crystalline array of nAChRs. Electron microscopy of these tubules has resulted in a 9 Å and a 4.6 Å resolution structure (Unwin, 1993; Miyazawa *et al.*, 1999). Viewed from the extracellular face, the pentameric structure is readily identifiable. The nAChR is roughly shaped as a cylinder 110 Å long and 70 Å in diameter. The extracellular portion of the cylinder is approximately 60 Å long, with a 20 Å diameter opening along the central axis. The intracellular portion is only 15 Å long, also including an opening 20 Å in diameter. The portion spanning the lipid bilayer is approximately 30 Å long, with no apparent opening but rather a constriction, indicating that this area may be the gate for the ion channel. The density of each subunit closest to the central axis appears to be α-helical, indicating a transmembrane segment lining the pore. This helix appears to have a kink near the middle of the bilayer, proposed to form the channel gate. A putative area for the agonist binding site has been proposed, approximately 30 Å above the lipid bilayer. However, full details of the structure are not available because the low resolution of the images does not allow a trace of the protein backbone.

LIGAND GATED ION CHANNELS AS ALLOSTERIC PROTEINS



The ligand-gated ion channels are each modulated allosterically by several types of drugs. Agonists cause a change in the distribution of states, shifting the equilibrium from the resting state (R) to the open state (O). Prolonged exposure to agonist converts the receptor to a desensitized state, characterized by high agonist affinity but lack of current. Two desensitized states have been observed, one (D_1), occurring within a second of agonist exposure, and the second one (D_2) occurring after seconds to minutes of exposure. Competitive antagonists block the binding of agonist and thereby prevent agonist from shifting the equilibrium to the open state. Other drugs, known as noncompetitive antagonists, allow agonist to bind but prevent the gating of the ion channel. This reduction in current flow could result from different actions of the drugs. For example, preferential binding in the channel could sterically occlude the channel, blocking the flow of current. Alternatively, a drug may stabilize the desensitized state of the receptor, reducing the amount of receptor in the activatible state. The response to agonist may also be potentiated by drugs, drugs that either stabilize the open state or destabilize the desensitized state.

At equilibrium, the permeability response of the receptor in the presence of agonist is not observed, due to the desensitization of the receptor. The binding of drugs can then be described approximately by a two state model:



where R is the resting state receptor, D is the receptor in the desensitized state, and A is the agonist (or antagonist) (Katz and Thesleff, 1957). This model has been used as a simple scheme with which to characterize the binding of many drugs to the nAChR. The initial ratio of resting to desensitized varies by cell type, with nAChR isolated from the *Torpedo* electric organ being ~10% desensitized (Boyd and Cohen, 1980a) while the nAChR expressed in BC3H-1 cells is ~0.01% desensitized (Sine and Taylor, 1982). nAChR agonists, such as ACh and carbamylcholine, have a higher affinity for the desensitized state than the resting state, causing a shift in the ratio to ~99% desensitized in *Torpedo*. d-Tubocurarine (dTC), a competitive antagonist, has a slight preference for the desensitized state, resulting in ~60% of the *Torpedo* nAChRs desensitized (Neubig and Cohen, 1979). α-Bungarotoxin (αBgTx), in contrast, does not cause an allosteric shift in the ratio of the states, but acts primarily to block the binding of agonist to the nAChR.

The binding of other cholinergic drugs, such as the noncompetitive antagonists (NCAs), to the nAChR can also be described using this two-state model. Three of these drugs, phencyclidine (PCP), histrionicotoxin (HTX), and tetracaine, have been shown to bind the nAChR with a stoichiometry of one drug per nAChR (Middleton *et al.*, 1999; Heidmann *et al.*, 1983). Similar to agonists and competitive antagonists, the binding of these drugs is dependent on the state of the nAChR. To determine the affinity of these drugs for the resting vs. desensitized state, agonists or competitive antagonists have been used to fix the conformation of the nAChR. PCP binds preferentially to the desensitized state, with a K_D of 0.8 μM in the presence of carbamylcholine. In the resting state, defined by the binding of PCP in the presence of αBgTx, PCP has a K_D of 5 μM

(Heidmann *et al.*, 1983). Tetracaine binds the resting state with higher affinity than the desensitized state, with a K_D of 0.5 μM in the absence and a K_D of 40 μM in the presence of carbamylcholine (Middleton *et al.*, 1999). At 20 °C, HTX binds both states with similar affinity (K_D=0.16 μM in the presence of carbamylcholine, 0.25 μM in the presence of αBgTx) (Heidmann *et al.*, 1983). At 4 °C, however, HTX has a higher affinity for the desensitized state (K_D=0.4 μM in the presence of carbamylcholine, 6 μM in the absence) (Boyd and Cohen, 1984). This model provides a rudimentary system with which to study the interactions of other drugs with the nAChR.

STRUCTURAL MOTIFS

In the absence of high resolution structural information, studies using affinity labeling and mutagenesis have yielded information on the overall structure of the receptors in the nAChR family. The main structural motifs identified are the agonist binding site, pore-forming region, and the protein-lipid interface. These regions appear homologous across the members of the family, at least in basic topology, as expected from their sequence similarities. The agonist binding sites of the receptors are composed of at least 6 loops at the interface of 2 subunits, both of which contribute 3 loops. Each loop contains residues conserved across all subunits, perhaps contributing to the structure of the loops. Of the four transmembrane segments, the M2 segment has been the most extensively studied, and it appears to form most of the ion channel. In the nAChR, evidence indicates that the M1 segment also contributes to the ion channel, while the M3 and M4 segments are present at the protein-lipid interface.

Agonist binding site

The agonist binding site of the nAChR consists of at least 6 loops at the subunit interfaces, with regions contributed by the α subunit and, in non-homomeric nAChRs, the

non-α subunit (the γ or δ subunit in *Torpedo* nAChR or embryonic muscle) (Figure 1-3). In the α subunit Loop A, Tyr-93 has been implicated in agonist and competitive antagonist binding by both affinity labeling and mutational studies (Sine *et al.*, 1994; Cohen *et al.*, 1991; Galzi *et al.*, 1990). In Loop B, Trp-149 and Tyr-151 are labeled by DDF, a reversible competitive antagonist (Dennis *et al.*, 1988). Mutations of Trp-149 have also implicated it as contributing to the agonist binding site (Zhong *et al.*, 1998). Several residues in Loop C contribute to agonist and competitive antagonist binding. Tyr-190, Cys-192, Cys-193, and Tyr-198 have each been implicated by photoaffinity labeling (Middleton and Cohen, 1991; Abramson *et al.*, 1989; Dennis *et al.*, 1988; Chiara and Cohen, 1997; Kao *et al.*, 1984). Mutations at Tyr-190, Tyr-198, and Asp-200, as well as several other residues in Loop C, affect the binding of a several agonists and antagonists (Tomaselli *et al.*, 1991; Aylwin and White, 1994; Sine *et al.*, 1994; Fu and Sine, 1994; O'Leary *et al.*, 1994; Ackermann and Taylor, 1997; Osaka *et al.*, 1998).

Loops D, E, and F are on the opposite side of the subunit interface, the γ or δ subunits in the *Torpedo* or embryonic muscle nAChR. Within Loop D, γTrp-55 and its homolog in δ, δTrp-57, contribute to agonist and antagonist binding, as shown by both affinity labeling and mutagenesis (Prince and Sine, 1996; Chiara and Cohen, 1997; Chiara *et al.*, 1998; O'Leary *et al.*, 1994; Corringer *et al.*, 1995). Loop E residues at positions γTyr-111, γMet-116, and γTyr-117, their homologs in δ, and γLeu-119 all contribute to the agonist binding site (Fu and Sine, 1994; Chiara *et al.*, 1999; Sine, 1993; Sine *et al.*, 1995). In loop F of the *Torpedo*, γAsp-174 and its homolog in δ affect the binding of agonists and antagonists. γSer-161 and γPhe-172 in mouse muscle, as well as their homologs in δ, also affect the binding of agonists and antagonists (Martin and Karlin, 1997; Osaka *et al.*, 1998; Czajkowski *et al.*, 1993; Sine, 1993; Sine *et al.*, 1995).

This multi-loop structure is also present in the $GABA_A$ agonist binding site. In the $GABA_A$ receptor, however, the agonist site is at the interface of β and α, where $GABA_A$ α plays a role similar to that of the γ or δ subunit of the nAChR. So far, Loops B, C, D, and E have been shown to contribute to the binding site. In Loop B, mutation of Tyr-157 or Thr-160 shifts agonist EC_{50} rightward (Amin and Weiss, 1993). Similarly, mutations of βThr-202 or βTyr-205 in Loop C cause a shift in response to GABA (Amin and Weiss, 1993). In Loop D, on the α subunit, α1 Phe-65 was implicated in agonist binding by both photoaffinity labeling and mutagenesis (Smith and Olsen, 1994; Buhr and Sigel, 1997). Mutation of α1Ile-121 in Loop E reduces the binding affinity and potency of agonist (Westh-Hansen *et al.*, 1997).

The glycine and $5HT_3$ receptor agonist sites also appear to consist of the multi-loop structure. Residues in loops B and C have been shown to contribute to the glycine site, while residues in Loop D have been shown to contribute to $5HT_3$ binding. In the glycine receptor α1 Loop B, mutation of Phe-159, Gly-160, or Tyr-161 alters the agonist binding properties of the receptor (Vandenberg *et al.*, 1992; Schmeiden *et al.*, 1993). In Loop C, mutations of residues at α1Lys-200 and Tyr-202 affect the binding of antagonists (Vandenberg *et al.*, 1992). In the $5HT_3$ receptor, residues Trp-89, Arg-91, and Tyr-93 of Loop E contribute to the binding affinity of agonists and competitive antagonists (Yan *et al.*, 1999).

Surprisingly, the binding site on the $GABA_AR$ of benzodiazepines also is highly homologous to the agonist binding sites on the nAChR family. Benzodiazepines potentiate the response to GABA but are unable to open the channels in the absence of agonist. The binding site of the benzodiazepines is on the α-γ interface, where α contributes loops similar to nAChR α, and γ is equivalent to the nAChR γ or δ. So far, all the loops except Loop F have been shown to contribute to this binding site. α1His-101

has been implicated in the benzodiazepine binding site on Loop A by both photoaffinity labeling and mutational analysis (Duncalfe *et al.*, 1996; Korpi *et al.*, 1993; Wieland *et al.*, 1992; Wieland and Lüddens, 1994). In Loop B of the $GABA_A$ α subunit, α1Tyr-159, α1Tyr-161, and α1Thr-162 affect the binding of benzodiazepines (Amin *et al.*, 1997; Buhr *et al.*, 1996; Wieland and Lüddens, 1994). The benzodiazepine binding site also has a contribution from Loop C, at residues Tyr-209, Thr-206, Ser-204, and Gly-200 (Amin *et al.*, 1997; Buhr *et al.*, 1997a; Buhr *et al.*, 1996; Wieland and Lüddens, 1994; Pritchett and Seeburg, 1991; Renard *et al.*, 1999). On the opposite side of the binding site, the equivalent of the non-α subunit of the nAChR, Loop D and Loop E of the $GABA_A$ γ subunit contribute to the benzodiazepine site. So far, the only residue in Loop D implicated in benzodiazepine binding is Phe-77, while in Loop E mutations at Met-130 affect the affinities of several benzodiazepines (Wingrove *et al.*, 1997; Buhr *et al.*, 1997b).

From a combination of the multiple mutagenesis and affinity labeling experiments, the basic contributions to the agonist binding sites have been established. However, many questions remain unanswered. For example, the position of the agonist site within the three-dimensional receptor structure is not well described, although some predictions of the location have been made by inspection of the electron micrograph images (Miyazawa *et al.*, 1999) and by fluorescence resonance energy transfer (Herz *et al.*, 1989; Valenzuela *et al.*, 1994). Additionally, a large number of residues have been implicated as contributing to the binding site, but it would be very difficult for so many to interact with all agonists, including one as small as trimethylammonium. Further studies of binding in the agonist site of all these receptors will be necessary to define the agonist sites.

Transmembrane segments

The subunits of the nAChR family each have four transmembrane segments, M1-M4. An understanding of the functional role and position of each has been elucidated with a variety of experiments, the majority on the nAChR. From the electron microscopy studies, the channel lining appears to be made up of an α helix contributed by each of the subunits. Based on photoaffinity labeling and electrophysiological data, this helix is the M2 segment. Additional evidence is beginning to show the participation of the M1 segment in channel formation as well. The M3 and M4 segments form the majority of the protein-lipid interface.

Ion Channel

The binding of agonist to the ligand gated ion channels causes a structural change that opens the ion pore. Although the $GABA_A$ and glycine receptors are permeant to anions while the nAChR and $5HT_3$ receptors are permeant to cations, structurally their pores appear similar. In each, the pore is lined primarily by one face of the M2 segment contributed by each subunit. There is also very strong evidence that common sites of the M2 helices compose the binding sites of at least some noncompetitive antagonists.

Mutagenesis studies with the muscle-type nAChR have illustrated some of the structural features of the M2 segment (Figure 1-4). A multiring structure formed by homologous amino acids contributed by all five subunits is predicted by many of these studies. (The residues in M2 are numbered from a homologous lysine or arginine in the N-terminus, as noted in Figure 1-4). The nature of the side chains in these rings also affects the current flow through the channel. Mutations at the C-terminal end of the M2 segments, position 20', result in an inward rectification, while mutations near the C-terminus, -5', result in an outward rectification. Additionally, mutations in these position

in all 4 subunits show similar, additive effects, suggesting a charge ring structure (Imoto *et al.*, 1988). At position 9', mutation of Leu to Ser decreased ACh EC_{50} by 10-fold. For each subsequent mutant subunit present, the shift in EC_{50} was additive, such that with 5 mutant subunits, the ACh EC_{50} was decreased by 10,000-fold (Labarca *et al.*, 1995). Additionally γLeu9'Thr showed a decrease in desensitization and stabilization of the open state (Filatov and White, 1995). At the 2' position, conductance is inversely related to the volume of the amino acid. It is possible that this ring forms the narrowest part of the open channel, determining the size of the ions that can flow through the channel (Villarroel *et al.*, 1991; Imoto *et al.*, 1991). Mutations in two other M2 residues, 12' and 15', have been linked to congenital myasthenic syndromes, with the 12' mutation resulting in prolonged openings in the presence of agonist (Ohno *et al.*, 1995; Gomez and Gammack, 1995). Most of the aforementioned residues, when mutated to cysteine, are all modified by water soluble probes, and that modification irreversibly alters the current in response to acetylcholine (Akabas *et al.*, 1994; Zhang and Karlin, 1998). The exposure of every third or fourth residue, along with the structural imaging data (Unwin, 1993), suggests an α-helical structure for the M2 segment.

The interaction of noncompetitive antagonists (NCAs) with the M2 segments provides additional evidence for its role as the ion pore. In single channel studies, the aromatic amine QX-222 is seen to block the current of the open channel but not allow the channel to close. The residency time of QX-222 is shorter than the open time of the channel, and each time QX-222 unbinds, current flows. This flickering suggests that QX-222 enters and leaves the channel rapidly, and its presence in the channel sterically prevents the transition to a closed or desensitized state (Neher, 1983). Additional evidence that QX-222 binds in the channel comes from mutational analysis. For example, mutations of residues at 6' from polar to nonpolar or residues at 10' from

nonpolar to polar reduces the potency of channel block by QX-222 (Charnet *et al.*, 1990; Leonard *et al.*, 1988). Additionally, QX-222 is capable of protecting cysteines at positions 13', 10', 9', 6', and 2' from reaction with a water soluble probe. Cysteines at the extracellular end of the channel, 16' and 20', however, are not protected (Pascual and Karlin, 1998). Evidence for the binding of several other amine NCAs near the M2 segment comes from affinity labeling studies, discussed later.

Mutations in M2 residues of the neuronal nAChR are also capable of dramatically altering the channel conductance. Mutations at the 13', 9', and 6' positions result in conducting desensitized states (Devillers-Thiery *et al.*, 1992; Revah *et al.*, 1991). In addition, certain mutations at position 9' abolish Ca^{++} conductance (Bertrand *et al.*, 1993). Mutation of 2 residues, -1' and 13', of the neuronal nAChR M2 segment to their corresponding residues in the $GABA_A$ and glycine receptors, along with the addition of a proline present in these receptors, is sufficient to convert the nAChR channel from cation to anion selective (Galzi *et al.*, 1992). NCAs also appear to bind near the M2 segment of neuronal nAChR since mutations at Leu-9' to Ser or Thr in the α7 nAChR suppresses the block by 20 μM QX-222 (Revah *et al.*, 1991).

In addition to the M2 segment, the N-terminal end of the M1 segment also appears to contribute to pore formation. Cysteine mutations in this region are accessible to modification by water soluble probes (Akabas and Karlin, 1995; Zhang and Karlin, 1997). On the other hand, several residues in the C-terminal end of α and δ, which were inaccessible to probes, are labeled by [^{125}I]TID in a manner consistent with exposure near the protein-lipid interface (Blanton and Cohen, 1994; White and Cohen, 1992). The effects of side chain size at position γCys-230 indicate that this position also is not exposed to the channel (Lo *et al.*, 1991). Therefore, it is likely that just the N-terminus of M1 contributes to the lumen of the channel, while the C-terminus is further removed.

Although the $GABA_A$ and glycine receptors are anion channels, they appear to function similarly to the M2 segment of the nAChR. Cysteine scanning mutagenesis of the full M2 segment of $GABA_A$ α1, as well as a portion of the $GABA_A$ γM2 segment, has shown that the water exposed residues are located in approximately the same positions as those of the nAChR (Xu and Akabas, 1996). Additionally, noncompetitive antagonists of glycine and $GABA_A$ receptors are affected by mutations in the M2 segment. For example, picrotoxin is affected by the exchange of the glycine β1 M2 sequence for the α1 M2 sequence at non-homologous residues. Partial block is produced by the exchange of the C-terminal portion, while complete block requires exchange of the entire M2 segment (Pribilla *et al.*, 1992). In the $GABA_A$ receptor, picrotoxin and cyclodienes no longer block current of a *Drosophila* $GABA_A$ receptor with an Ala3'Ser mutation (ffrench-Constant *et al.*, 1993). Picrotoxin prevents the modification of $GABA_A$ α1Val2'Cys by cysteine modifying reagents, while α1Thr6'Cys is still modified in the presence of picrotoxin (Xu *et al.*, 1995). In agreement with picrotoxin binding near the N-terminal, most intracellular end of the $GABA_A$ M2 segment, mutations at β1Thr12'Gln allow wild-type block by both picrotoxin and penicillin (Birnir *et al.*, 1997). Another NCA of the $GABA_A$ receptor, Zn^{++} is affected by residues at the C-terminal end of the M2 segment. A mutation of β1His-17' to Ser, the homologous residue in α1, reduces the IC_{50} of Zn^{++} block by 300 fold, while the converse mutant increases the block by Zn^{++} (Horenstein and Akabas, 1998).

Protein-lipid Interface

The structure and location of the M3 and M4 transmembrane segments have been studied by photoaffinity labeling. Although in the three-dimensional structure determined from electron microscopy of the nAChR the only apparent α-helical content of the nAChR is the M2 segments, the pattern of photoaffinity labeling of the M3 and M4

segments suggests an α-helical structure (Figure 1-5). [^{125}I]TID, as well as [^{3}H]DAF, photoaffinity labeled several residues on the M4 segment in approximately an every-third pattern, consistent with an α-helix (Blanton and Cohen, 1992; Blanton and Cohen, 1994; Blanton *et al.*, 1998a). [^{125}I]TID labeled M3 in a similar manner (Blanton and Cohen, 1994). The incorporation of these hydrophobic drugs into the M3 and M4 segments was not affected by other cholinergic ligands, and these residues are expected to line the protein-lipid interface.

Although the nAChR is functional when the entire M4 segment is exchanged for a transmembrane segment from other membrane proteins (Tobimatsu *et al.*, 1987), particular residues do have dramatic effects on nAChR function. Mutation of αCys-418 to a variety of residues resulted in prolonged open channel lifetime, an increase of ~28-fold when mutated to tryptophan (Li *et al.*, 1992; Bouzat *et al.*, 1998; Lee *et al.*, 1994; Lasalde *et al.*, 1996; Ortiz-Miranda *et al.*, 1997). Similar effects were seen with mutations of αThr-422 (Bouzat *et al.*, 1998). Additionally, in the mouse nAChR, the nature of the residues at two positions on the γ subunit, at 440 and 442 (homologous to αCys-412 and αPhe-414 of *Torpedo*), alters the nAChR channel kinetics (Bouzat *et al.*, 1994). The composition of the M3 segment has also been shown to affect the gating of the nAChR (Campos-Caro *et al.*, 1997). These mutation illustrate the highly allosteric nature of the nAChR, indicating that modification as far removed from the channel or agonist binding site as the protein-lipid interface can alter the gating.

NONCOMPETITIVE ANTAGONISTS

The noncompetitive antagonists (NCAs) are a structurally diverse group of compounds that reversibly inhibit current through the channel without blocking the binding of agonist (Figure 1-6). As discussed with regards to the M2 transmembrane segment, a simple view of the binding of certain NCAs is pore occlusion. Most aromatic

amine NCAs appear to bind in the lumen of the channel. Other classes of drugs, such as the general anesthetics, have a currently unknown site or sites of action.

Amine NCAs

Several drugs of the aromatic amine family act as NCAs on the nAChR. Photoaffinity labeling studies with many of these have shown that their primary site of binding is the M2 segment, in the lumen of the channel. Of the compounds known to bind one molecule per nAChR, only the binding site of tetracaine has been determined, while the sites of binding of HTX and PCP remain unclear. [^{3}H]Tetracaine, which binds preferentially to the resting state, labels residue 9' in all subunits as well at 13' in α and δ (Gallagher and Cohen, 1999). In the presence of carbamylcholine, [^{3}H]chlorpromazine (CPZ) labels residues in all four subunits, including Ser-6' in each (Giraudat *et al.*, 1986; Giraudat *et al.*, 1987; Giraudat *et al.*, 1989; Revah *et al.*, 1990). [^{3}H]Triphenylphosphonium (TPP) also labels this residue in all the subunits except γ (Hucho *et al.*, 1986). The binding site of meproadifen is slightly removed from the binding sites of the others, with affinity incorporation of [^{3}H]meproadifen mustard into Glu-20' (Pedersen and Cohen, 1990). These results suggest that these noncompetitive antagonists bind in the pore, which is formed by the M2 segments of all of the subunits, and most access the subunits equally.

Two uncharged aromatic NCAs, [^{125}I]TID and [^{3}H]DAF, label similar regions to the aforementioned aromatic amine NCAs. However, unlike the labeling of these drugs in M4, the labeling in M2 is inhibitable and affected by cholinergic drugs. In the presence of agonist, [^{125}I]TID labels residues at 6', as well as at 2', 9', and 13'. In the absence of agonist, however, [^{125}I]TID only labels 9' and 13' (White and Cohen, 1992). A derivative of TID, [^{125}]I-TID-BE labels βL9', βV13', and βL16' in the presence of carbamylcholine (Blanton *et al.*, 1998b). [^{3}H]DAF labels βV13' and δV13' in the

absence of carbamylcholine, with incorporation inhibitable by tetracaine; in the presence of carbamylcholine, incorporation is present at βL9', βA10', δS6' and δL9', with the incorporation inhibitable by PCP, another NCA (Blanton *et al.*, 1998a).

There is some evidence that not all aromatic amines bind in the M2 domain. Quinacrine binds competitively with [^{3}H]PCP, but the photoactivatible probe [^{3}H]quinacrine azide incorporates into the N-terminus of the M1 segment in the open state (DiPaola *et al.*, 1990; Karlin, 1991). Additionally, mutations in this region affect the block by quinacrine (Tamamizu *et al.*, 1995). The effect of mutations in the M2 segment on the block by quinacrine has yet to be reported. These M1 residues, when mutated to cysteine, are accessible to a water soluble probe, and most likely contribute to the channel lumen (Akabas and Karlin, 1995; Zhang and Karlin, 1997).

The binding sites of other aromatic amine NCAs have been assumed to be near that identified for the photoaffinity drugs. For example, PCP and HTX, in most cases, competitively inhibit the binding of other aromatic amines, although their sites of binding are unknown. Competitive binding of a given drug with HTX or PCP has been used as a simple approximation of binding in the lumen of the channel. However, in certain cases the results challenge this assumption. For example, in the desensitized state, TID fully inhibits the binding of [^{3}H]PCP and allosterically inhibits the binding of [^{3}H]HTX. In the resting state, though, TID again allosterically inhibits [^{3}H]HTX binding, but does not affect the binding of [^{3}H]PCP (White *et al.*, 1991). Additionally, mutations in M1 which affect quinacrine inhibition have no effect on the inhibition by CPZ (Tamamizu *et al.*, 1995).

Ethidium is another aromatic amine NCA that inhibits the binding of PCP and HTX. At equilibrium in the presence of agonist, ethidium inhibits the binding of [^{3}H]PCP with a K_D of 0.4-0.6 μM (Herz *et al.*, 1987; Pedersen, 1995). The inherent

fluorescence of ethidium makes it a good tool for studies of the structure of the nAChR. Ethidium has been used as a probe in measurements of channel kinetics, with fewer complications than the more traditional flux assays (Rankin *et al.*, 1997). Initial studies using fluorescence resonance energy transfer (FRET) showed that the distance between the ethidium NCA binding site, which was presumed to be the channel lumen, and the agonist site, using fluorescent agonist and competitive antagonist, was ~30 Å (Herz *et al.*, 1989) (Figure 1-7). However, subsequent FRET experiments measured the distance between the ethidium NCA site and the lipid membranes as 50 Å, placing the binding site of ethidium at the most extracellular end of the nAChR (Johnson and Nuss, 1994). This distinct site of binding was unexpected, due to the structural similarity to other aromatic amine NCA. If the ethidium binding site is removed from the channel, the mechanism of action, as well as many assumptions based on competitive binding of drugs, will need to be reconsidered.

Anesthetics

Anesthetics are another class of drugs which act as NCAs on the nAChR, and they also modulate the $GABA_A$ and glycine receptors. The wide range of structures that cause anesthesia, from small halogenated hydrocarbons to long-chain alcohols to steroids, led earlier researchers to propose that the drugs act by a nonspecific mechanism, probably disordering of the lipids. This theory has been supported by a comparison of the potency of various general anesthetics to their lipid partition coefficient, the Meyer-Overton correlation. However, accumulating evidence suggests that general anesthetics are likely to exert their effects by direct interactions with receptors and other proteins (Franks and Lieb, 1994). Several studies have been aimed at determining if there are specific binding sites for general anesthetics on the ligand gated ion channels.

Alcohols

One class of anesthetics that has been shown to affect the nAChR family is the alcohols. Evidence for a specific site/sites of action comes from the effects of varying molecule size as well as from interaction between alcohols inhibiting the nAChR. The alcohols have a size cut-off effect, since alcohols longer than dodecanol have no effect on the nAChR (Alifimoff *et al.*, 1989). Based on additional studies with cycloalkanemethanols, the cut off for inhibition is related to the volume, and not the length, of the molecule (Wood *et al.*, 1993).

The size of the molecule affects not only whether the alcohol has an effect, but also the nature of the effect. In flux assays with the nAChR, ethanol shifts the ACh K_A (the 50% activating concentration) leftward, apparently increasing the ACh affinity, and speeds the desensitization at low concentrations of agonist (Forman and Miller, 1989; Wood *et al.*, 1991). Larger alcohols, such as octanol and hexanol, reduce the maximum flux through the channels without changing agonist affinity (Wood *et al.*, 1991). At low agonist concentrations, propanol and butanol enhance flux, as does ethanol, while at high concentrations they inhibit flux similarly to octanol. When octanol and ethanol are applied together, the maximum flux is decreased, as with octanol alone, and the K_A of ACh is shifted, as with ethanol, suggesting that the two compounds act at different sites (Wood *et al.*, 1991). Other flux assays suggest that octanol and heptanol act in a mutually exclusive manner, probably binding to the same site (Wood *et al.*, 1995).

Unlike muscle nAChR, the α7 nAChR is inhibited by ethanol, and the $5HT_3$ receptor is potentiated by ethanol. A chimera between these two receptors, with the N-terminal 200 amino acids from the α7 nAChR, was inhibited by ethanol, showing that ethanol exerts its effects on the nAChR N-terminal to the M2 segment (Yu *et al.*, 1996). Efforts to map the site of action of the long chain alcohols, however, have implicated the

M2 segment of the nAChR (Figure 1-8). Mutation of αSer-10'Ile increases the sensitivity of the nAChR to block by octanol. As this side chain is varied, the more hydrophobic side chains increase sensitivity to hexanol. The double mutant, αSer-10'Ile and βThr-10'Ile, increases the sensitivity to hexanol more than αSer-10'Ile alone (Forman *et al.*, 1995). However, the βThr-10'Ile, has less of an effect on the sensitivity to octanol or hexanol than αSer-10'Ile, and both affect the sensitivity more than mutations in γ10' or δ10'. These mutations do, however, have additive effects on sensitivity to alcohol block (Forman, 1997). At least one mutation in the 6' position, αSer-6'Phe, does not affect the sensitivity to alcohols as much as the mutations at the 10' position, suggesting that the alcohols do not bind on the more intracellular side of the channel (Forman *et al.*, 1995).

Unlike their actions on the nAChR, alcohols potentiate the response in the $GABA_A$ and glycine receptors, at least with some isoforms. Using mutagenesis, several experiments have addressed the location of the alcohol binding site (Ye *et al.*, 1998; Wick *et al.*, 1998; Mihic *et al.*, 1997). These studies have implicated two residues, one in the M2 segment and one in M3. The residue in the M2 segment, 15', is predicted to lie on the face of the α-helix pointing away from the channel lining. The position of the M3 residue is less well defined, but has been predicted to lie on the face of M3 adjacent to the M2 helix, at approximately the same level as the M2 residue (Wick *et al.*, 1998).The size of the residues at these positions affects the cut-off of the alcohols on these receptors. In addition to alcohols, substitutions at these residues also alter the potentiation by volatile general anesthetics, isoflurane and enflurane, as well as intravenous anesthetics, loreclezole and etomidate (Krasowski *et al.*, 1998; Mihic *et al.*, 1997; Wingrove *et al.*, 1994; McGurk *et al.*, 1998; Moody *et al.*, 1997; Belelli *et al.*, 1997).

The data from the nAChR and $GABA_A$ and glycine receptor implicate different portions of the M2 segment as the site of action of alcohols. It is possible that the different receptors have distinct binding sites for alcohols. However, either mutation may affect the action of the alcohols allosterically. For example, in the nAChR the αSer-10'Ile mutant effectively converts ACh to a partial agonist. This effect alters the observed action of ethanol, although it is not expected that ethanol binds to this site (Forman and Zhou, 1999). An alternative approach, like photoaffinity labeling, could help pinpoint the site of action of the alcohols.

Steroids

Steroids, another class of anesthetics, also modulate the response of nAChR family receptors. The high lipophilicity of steroids suggests a possible interaction at the protein-lipid interface. However, electrophysiological experiments with the nAChR have provided evidence that the steroids do not act via the protein-lipid interface. Binding to a site accessible only from the extracellular side is supported by a decrease in inhibition when hydrocortisone and 11-deoxycortisone are applied intracellularly (Bouzat and Barrantes, 1993). Experiments with a modified progesterone coupled to bovine serum albumin (BSA) show that even a strongly hydrophilic steroid could still inhibit current, suggesting that the steroid does not need to enter the lipid bilayer to exert its effects (Valera *et al.*, 1992; Ke and Lukas, 1999).

The action of steroids is different from other NCAs, suggesting the existence of a distinct site of binding. When progesterone is applied before agonist, and then washed off, the current is still inhibited (Valera *et al.*, 1992). Hydrocortisone and 11-deoxycortisone cause a decrease in burst duration, indicating that the channel may be able to adopt a closed conformation while still being bound by the steroid (Bouzat and

Barrantes, 1993). Additionally, the application of hydrocortisone with QX-222 alters the single channel properties from those seen with QX-222 alone, suggesting that the two drugs bind different sites (Bouzat and Barrantes, 1996). This data suggests that steroids are not traditional channel-blockers, if they even bind in the channel at all.

As with the nAChR, the site of action of steroids on the $GABA_A$ receptor is unknown. The site of interaction is not at any of the traditional binding sites, such as the agonist, barbiturate, or benzodiazepine sites. A variety of steroids enhance agonist and benzodiazepine binding to the $GABA_A$ receptor, and steroids still enhance current in the presence of barbiturates (Paul and Purdy, 1992). Chimeric proteins constructed from the glycine $\alpha 1$ subunit and the $GABA_A$ $\alpha 2$ or $\beta 1$ subunits indicate the site of action of steroids is N-terminal to the middle of the M2 segment (Rick *et al.*, 1998). Mutations in residues implicated in the binding of alcohols and small molecule anesthetics show no effect on the action of neurosteroids, providing additional evidence that they bind an as-yet undiscovered site (Belelli *et al.*, 1997; McGurk *et al.*, 1998).

Based on photoaffinity labeling studies on the nAChR with [^{3}H]promegestone, steroids bind near the protein-lipid interface, at least. [^{3}H]Promegestone photoincorporated into residues in M4 previously identified by [^{125}I]TID as forming the protein-lipid interface (Blanton *et al.*, 1999). However, lack of labeling in the channel could be explained by poor reactivity of the photoactivatible group with the channel sidechains or the positioning of the group far from any sidechains. Further work is necessary to determine clearly the site of steroid action.

PHOTOAFFINITY LABELING

While much of the data regarding the functional domains of the receptors comes from mutagenesis studies, there are complications in interpreting results from these

experiments. For example, a mutation in one area may cause an allosteric change in a distant area, as with the M4 mutant that alters channel gating (Li *et al.*, 1992). These changes may also affect the binding of drugs. A mutation may cause a structural change undetectable by agonist response, but affecting the response to another drug. Alternatively, a mutation may alter the response to agonist and thereby alter the effect of another drug. The αSer-10'Ile mutation causes such a change in nAChR response to ethanol (Forman and Zhou, 1999).

To complement mutational analyses, photoaffinity labeling potentially provides a means of identifying amino acids contributing to the drug binding site. As described above, this method has been used extensively with the nAChR to map the agonist binding site and also to identify the binding sites of aromatic amines within the ion channel. In these studies, photoaffinity labeling and mutational studies showed complementary results. Photoaffinity labeling, however, also is problematic. Ideally, the photoactivatible compound has a similar structure to the drug of interest, possibly being the drug itself. Additionally, the compound should bind similarly to, and have the same pharmacological effects as, the drug of interest. Photolysis should be carried out at a UV wavelength that does not damage the protein. The photolysis should generate a reactive group that reacts rapidly and efficiently, with no selectivity for the nature of the neighboring side chain. The adduct formed should be robust to conditions used in analysis, such as HPLC and sequencing. In reality, most photoaffinity probes do not meet all of these criteria.

The aryl azides are the most commonly used photoaffinity probes, due to their ease of synthesis. However, they can display selective reactivity, and the adducts formed are often not stable. When aryl azides are photolyzed, they form reactive singlet nitrenes, which, unfortunately, readily rearrange to ketenimine azepines (Figure 1-9). The nitrenes have a broad reactivity while the azepine reacts preferentially with nucleophiles such as

cysteines. Although cysteines have been labeled predominantly by aryl azide ligands, other, less nucleophilic amino acids have also been shown to react with aryl azides (Fleming, 1995; Kotzyba-Hibert *et al.*, 1995). One other disadvantage of the aryl azide photochemistry is that the photoadducts formed between the ketenimine azepines and glutamic and aspartic acids are very sensitive to hydrolysis, and adducts with some other amino acids are also slightly sensitive (Bayley, 1983). In addition, aryl azides must be photolyzed at 254 nm, a wavelength that can damage protein.

Aryl azide derivatives of several nAChR NCAs have been synthesized (Figure 1-10). For example, progestin aryl azide was synthesized as a probe of the progesterone receptor (Kym *et al.*, 1995). This compound binds the progesterone receptor with high affinity, and shows high photoincorporation efficiency, with approximately 60% of the receptor labeled with high specificity. Since progesterone acts as a NCA on the nAChR, this compound could be useful in localizing the site of steroid binding on the nAChR. [^{3}H]Quinacrine azide, which has been used as a photoaffinity probe of the nAChR, is also an aryl azide (DiPaola *et al.*, 1990). A photoactivatible derivative of ethidium, ethidium diazide, has also been synthesized for studying the nAChR (Witzemann and Raftery, 1978; Pedersen, 1995). This drug binds the nAChR similarly to ethidium and photoincorporates into several subunits. Further studies with this compound will help localize the site of ethidium binding on the nAChR.

Another photoactivatible group, the diazirine, shows less selectivity and higher reactivity than the aryl azides. One aromatic diazirine, (trifluoromethyl)phenyldiazirine, for example, shows broad reactivity to side chains, and is photolyzed at 365 nm, a wavelength that is not expected to cause changes in the protein. This group has been used successfully with the nAChR as [^{125}I]TID. [^{125}I]TID labeled a wide variety of side

chains, including some with which it is expected to have low reactivity (Blanton and Cohen, 1992; Blanton and Cohen, 1994; White and Cohen, 1992). Aromatic diazirines, however, are not used as frequently as the aryl azides, due to difficulties of synthesis of the diazirine.

An alternative to the aromatic diazirine is the aliphatic diazirine. Although the aliphatic carbene generated upon photolysis is not as well stabilized as the aromatic carbene, aliphatic diazirines have been useful in photoaffinity studies. Examples of aliphatic diazirines include several oligosaccharides as well as a fatty acid (Schmider *et al.*, 1996; Liessem *et al.*, 1995). However, most reports have not been able to clearly identify the labeled amino acids by sequence analysis, though one report has determined the labeled residue by alternative methods (Liessem *et al.*, 1995). Another aliphatic diazirine that has been developed is 3-azioctanol (Husain *et al.*, 1999). This compound has been shown to have similar pharmacological properties to octanol. In tadpoles it induces anesthesia with an EC_{50} of ~160 μM, between the potency of heptanol and octanol. Additionally, 3-azioctanol potentiates the current in $GABA_A$ receptors, while inhibiting the current in nAChR, similarly to octanol. This compound will be useful in localizing the site of action of long chain alcohols on the nAChR and, potentially, on the $GABA_A$ receptor.

THESIS SYNOPSIS

This dissertation consists of experimental work with three different nAChR NCAs, each presented in a separate chapter. The localization of the binding site of [^{3}H]ethidium diazide is presented in Chapter 2. Based on homology to other aromatic amines, the binding site of ethidium might be expected to be in the nAChR pore. However, as described above, experiments with fluorescence resonance energy transfer indicated a binding site near the most extracellular region of the nAChR. [^{3}H]Ethidium

diazide has been previously shown to photoincorporate into nAChR (Witzemann and Raftery, 1978; Pedersen, 1995) and was used it here to map the site of ethidium binding. nAChR subunits labeled with [^{3}H]ethidium diazide were isolated by SDS-PAGE and subjected to proteolytic digestion and Edman degradation to identify the sites of incorporation. The experiments showed that the high affinity binding site of ethidium in the nAChR in the desensitized state is within the lumen of the channel, with contributions from both the M2 and M1 segments.

In Chapter 3, the results of photoincorporation of [^{3}H]3-azioctanol are presented. Evidence from mutational studies with the nAChR and $GABA_AR$ has implicated the M2 segment as contributing to the sites of action of long chain alcohols on these receptors. However, the regions identified in the two receptors are on different faces of the M2 helix. Here, the photoactivatible alcohol [^{3}H]3-azioctanol was photoincorporated into the nAChR. The sites of incorporation were mapped using proteolytic digestion and Edman degradation in an attempt to clarify the site of binding of long chain alcohols on the nAChR. The primary site of [^{3}H]3-azioctanol incorporation in the nAChR in the desensitized state was αGlu-262, at the extracellular end of M2, indicating binding within the lumen of the channel.

Preliminary studies using the photoactivatible steroid [^{3}H]progestin aryl azide as a probe of the steroid binding site on the nAChR are presented in Chapter 4. Previous reports on the incorporation of a photoactivatible steroid, [^{3}H]promegestone, showed incorporation in the M4 segment. To extend this analysis, I used a structurally related steroid, [^{3}H]progestin aryl azide, which contained a different photoactivatible group, to determine if another steroid labeled M4 or other regions of the nAChR. Labeled subunits were subjected to proteolytic digestion and Edman degradation to determine labeled regions. Although the instability of the photoadducts to HPLC and sequencing conditions,

as well as the poor recovery of peptide fragments, precluded the identification of labeled residues, the primary site of [^{3}H]progestin aryl azide incorporation in the α-subunit was within αM4.