Lysine Scanning Mutagenesis Delineates Structural Model of the Nicotinic Receptor Ligand Binding Domain

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Summary-

Nicotinic acetylcholine receptors (AChR) and their relatives mediate rapid chemical transmission throughout the nervous system, yet their atomic structures remain elusive. Here we use lysine-scanning mutagenesis to determine the orientation of residue side chains toward core hydrophobic or surface hydrophilic environments, and use this information to build a structural model of the ligand binding region of the AChR from adult human muscle. The resulting side chain orientations allow assignment of residue equivalence between AChR subunits and an acetylcholine binding protein (AChBP) solved by x-ray crystallography, providing the foundation for homology modeling. The resulting structural model of the AChR provides a picture of the ACh binding site and predicts novel pairs of residues that stabilize subunit interfaces. The overall results suggest that lysine scanning can provide the basis for structural modeling of other members of the AChR superfamily, as well as of other proteins with repeating structures delimiting a hydrophobic core.
Introduction-

Traditional methods for atomic structural determination use x-ray crystallography or NMR spectroscopy. However many proteins do not form crystals and many are too large to solve by NMR. New methods are therefore urgently needed to determine structures of such intractable proteins. Here we develop a mutagenesis-based modeling method and apply it to the ligand binding region of the nicotinic AChR from adult human muscle. The method uses lysine scanning to distinguish core hydrophobic from surface hydrophilic orientations of residue side chains, and uses this information to align residues in AChR subunits with equivalent residues in the homologous AChBP, which was solved by x-ray crystallography (1). The experimentally determined alignment forms the foundation for generating an atomic structural model of the ligand binding region of the heteromeric AChR.

Knowledge of nicotinic receptor structure advanced along two independent lines of investigation over the past decade. The first line stemmed from primary sequence data deduced from cloning AChR subunits and their relatives (2). These studies included prediction of membrane spanning regions using hydropathy analysis, prediction of secondary structure from the sequence data (3) and identification of key residues by affinity labeling together with microsequencing, and by site-directed mutagenesis combined with functional measurements (4-6). The emerging picture of the AChR indicated a pentamer in which about half of each subunit contributes to an N-terminal extracellular domain that harbors the ACh binding sites. The remainder of each subunit consists of four transmembrane domains, the second of which contributes to the ion channel, and a large cytoplasmic domain between the third and fourth transmembrane domains. Mutagenesis and site-directed labeling localized the ACh binding sites to interfaces between α and non-α subunits (4, 6), while studies of residue accessibility and residues affecting ion permeability revealed the channel gate and ion selectivity filter within the second transmembrane domain (7, 8).
Advances along the second line of investigation came from cryo-electron microscopy of two-dimensional arrays of AChRs from Torpedo. Data at 9 Å resolution revealed the overall shape and dimensions of the AChR, and identified secondary structural elements in the vicinity of the ACh binding site and the ion channel (9). The most recent data at 4.6 Å resolution revealed several aligned β strands near the putative ACh binding site, and a fenestrated basket-like structure extending into the cytoplasm (10). Images obtained following rapid application of ACh revealed that each subunit twists about the axis normal to the membrane when the ion channel opens (11). Overall, the cryo-electron microscopy data could be reconciled with the mutagenesis, labeling and functional measurements, although the precise location of the ACh binding sites and channel gate remained controversial.

Atomic structural insight recently emerged from the crystal structure of AChBP, a 120 kD acetylcholine binding protein homologous to the ligand binding region of the AChR (1). The sequence of AChBP is 23.9% identical to that of the homomeric α7 AChR, and harbors residues considered diagnostic of nicotinic receptor alpha subunits, including disulfide bonded cysteines and aromatic residues that contribute to the ACh binding site. These conserved elements suggest that AChBP may provide a model for the three dimensional structure of the ligand binding domain of the AChR and its relatives.

Here we combine lysine scanning mutagenesis, ligand binding measurements and homology modeling to deduce a structural model of the ligand binding region of the nicotinic AChR from adult human muscle. Lysine scanning of the ε subunit reveals that it oligomerizes with complementary AChR subunits when lysine is placed at alternating positions along the protein chain, indicating the presence of β strands and establishing orientation of the side chains toward core hydrophobic and surface hydrophilic environments. The side chain orientations allow alignment of equivalent residues between the ε subunit and AChBP, forming the foundation for homology modeling. The resulting
atomic structural model provides a detailed picture of the AChR ligand binding site and discloses novel residue pairs that stabilize subunit interfaces.
Experimental Procedures-

Materials- $^{125}$I-labeled $\alpha$-bungarotoxin (bgt) was from Dupont NEN, $d$-tubocurarine chloride from ICN Pharmaceuticals, Inc., 293 human embryonic kidney cells (293 HEK) from the American Type Culture Collection, $\alpha$-conotoxin GI from Sigma, and the fully methylated analog of $d$-tubocurarine, metocurine iodide, was a gift from the Eli Lilly Co.

Plasmids and Mutagenesis- Human adult AChR subunit cDNAs were obtained as described (12), and subcloned into the cytomegalovirus-based expression vector, pRBG4, as described (13). Mutations were constructed using the QuickChange kit from Stratagene. All mutations were confirmed by dideoxy sequencing.

Expression of mutant receptors and ligand binding measurements- HEK cells were transfected with mutant or wild type AChR subunit cDNAs using calcium phosphate precipitation as described (13). In all experiments, AChR subunit cDNAs were co-transfected in the following quantities per 10 cm plate of HEK cells: $\alpha$ (13.6 $\mu$g) and $\beta$, $\epsilon$ and $\delta$ (6.8 $\mu$g). Three days after transfection, intact HEK cells were harvested by gentle agitation in phosphate-buffered saline plus 5 mM EDTA. Ligand binding to intact cells was measured by competition against the initial rate of $^{125}$I-$\alpha$-bungarotoxin ($\alpha$-bgt) binding (14). After harvesting, the cells were briefly centrifuged, resuspended in high potassium Ringer’s solution, and divided into aliquots for ligand binding measurements. Potassium Ringer's solution contains: 140 mM KCl, 5.4 mM NaCl, 1.8 mM CaCl$_2$, 1.7 mM MgCl$_2$, 25 mM HEPES, 30 mg/liter bovine serum albumin, adjusted to pH 7.4 with 10-11 mM NaOH. Specified concentrations of competing ligand were added 30 minutes prior to adding $^{125}$I-$\alpha$-bgt, which was allowed to bind for 30 minutes to occupy approximately half of the surface receptors. Binding was terminated by addition of 2 ml of potassium Ringer’s solution containing 100 $\mu$M $d$-tubocurarine chloride. Cells were immediately
filtered through Whatman GF-B filters using a Brandel Cell Harvester and washed five times with 2 ml of potassium Ringer’s solution. Prior to use, filters were soaked in water containing 4% dried skim milk for at least two hours. Nonspecific binding was determined in the presence of 1 mM \(d\)-tubocurarine. The total number of \(\alpha\)-bgt binding sites was determined by incubation with the toxin for 90 minutes. The initial rate of \(\alpha\)-bgt binding was calculated as described (14) to yield fractional ligand occupancy. Competition measurements were analyzed according to one of the following equations: the Hill equation (Eq. 1), the sum of two binding sites present in equal numbers (Eq. 2), one binding site plus a constant (Eq. 3), the sum of two binding sites present in unequal numbers (Eq. 4) and weighted contributions of Eqs. 2 and 3 (Eq. 5):

\[
1-Y = \frac{1}{1 + ([\text{ligand}] / K_{\text{App}})^n} \quad \text{Eq. 1}
\]

\[
1-Y = 0.5/(1 + [\text{ligand}] / K_A) + 0.5/(1 + [\text{ligand}] / K_B) \quad \text{Eq. 2}
\]

\[
1-Y = \text{Fract}_A (1/(1 + [\text{ligand}] / K_A)) + (1-\text{Fract}_A) \quad \text{Eq. 3}
\]

\[
1-Y = \text{Fract}_A (1/(1 + [\text{ligand}] / K_A)) + (1-\text{Fract}_A)(1/(1 + [\text{ligand}] / K_B)) \quad \text{Eq. 4}
\]

\[
1-Y = \text{Fract}_{2\text{-site}} (1/(1 + [\text{ligand}] / K_A) + 1/(1 + [\text{ligand}] / K_B)) + (1-\text{Fract}_{2\text{-site}})(\text{Fract}_A/(1 + [\text{ligand}] / K_A) + (1-\text{Fract}_A)) \quad \text{Eq. 5}
\]

\(Y\) is fractional ligand occupancy, \(n\) is the Hill coefficient, \(K_{\text{App}}\) is an apparent dissociation constant, \(n\) is the Hill coefficient, \(K_A\) and \(K_B\) are intrinsic dissociation constants, \(\text{Fract}_A\) is the fraction of sites with dissociation constant \(K_A\) and \(\text{Fract}_{2\text{-site}}\) is the fraction of binding sites with equal numbers of \(K_A\) and \(K_B\) sites.

**Homology modeling** - We generated a homology model of the major extracellular domain of the adult human AChR using version 6.0 of the program MODELER (15), together with spatial restraints provided by the AChBP structure (1). We aligned AChR and AChBP sequences based on side chain dispositions determined by lysine scanning combined with ligand binding.
measurements, as described in Results (Table 1; Fig. 4). However to model strand β1, spatial restraints were removed for AChR εL40 and two flanking residues to account for its surface exposure (see Fig. 5), which is contrary to the corresponding aligned residue, I38, in AChBP. To maintain complementarity between subunits at their interfaces, all five subunits were modeled simultaneously. AChR α subunits were matched to A and C subunits of AChBP, and the ε, δ and β subunits were matched with the B, D and E subunits, respectively. We used the ‘patch’ command in MODELER to constrain coordinates of cystines 128 and 142, which form a disulfide bond in each subunit. Among several options in MODELER, we selected the ‘refine1 mode’, which generates the highest level of refinement using conjugate gradients coupled with simulated annealing and molecular dynamics. Modeling included all polar hydrogens to allow for main chain hydrogen bonding, but omitted non-polar hydrogens. We used MODELER to generate 100 different structures, and evaluated each structure using the programs PROCHECK (34) and PROFILES-3D (InsightII, MSI). Among the 100 structures, approximately 10 were of high quality and very similar, and we selected the best of these for further modeling.

The major insertions requiring additional modeling were the linkers between strands β8 and β9 in β, ε and δ subunits, which contain eight, eight and eleven inserted residues, respectively. The α subunit contains only one insertion in this linker, which MODELER is designed to accommodate (17). To model the β8-β9 linker in the ε subunit, a second round of MODELER was employed using different sequence alignments in this region, and constraining coordinates of all atoms except those of the β8-β9 linker. This was an empirical process, and we selected structures that satisfied several criteria. First, we required εF160 in this linker, and equivalent residues in other subunits, to project into the hydrophobic core to account for the observation that εF160K produced predominantly misfolded subunits (Table 2). Second, we
selected structures in which endogenous positively charged residues in the linker project away from the hydrophobic interior; these included εK171 εK167, δK164, δK167 and δR170. Third, we required εD175 and δD180 to approach within 10-15 Å of αC192/193 determined in cross linking experiments (16), and also within 15-20 Å of εL119 or δL121 determined from double mutant cycles analysis of α-neurotoxin binding (18). Although these constraints are qualitative, in practice they eliminated many candidate structures. PROCHECK and PROFILES 3-D were used to select the best structure for further modeling.

Finally, we applied two rounds of energy minimization using the program CHARMM (19), version 27b4. The first round constrained the coordinates of all heavy atoms, while allowing movement of hydrogens. The second round constrained the protein main chain, while allowing movement of side chains. The final structure was selected based on evaluations using PROCHECK and PROFILES 3-D.
Results-

*Lysine scanning mutagenesis to assess AChR secondary structure*

We mutated individual residues to lysine along a putative β strand from positions 49 through 61 of the ε subunit from adult human muscle AChR. Each lysine mutation was co-transfected with complementary α, β and δ subunit cDNAs in 293 HEK cells, followed by quantitation of cell-surface receptors using the AChR-specific ligand $^{125}$I-α-bgt. The results reveal a pattern of high expression alternating with low expression as lysine is advanced along the protein chain (Fig. 1a). High expression for odd numbered residues approaches that for wild type AChR, whereas low expression for even numbered residues approaches that for subunit-omitted $\alpha_2\beta\delta_2$ receptors. High expression likely results from mutant ε subunits that fold and incorporate into pentameric AChRs, whereas low expression likely results from misfolding of the mutant ε subunits, leading to subunit-omitted $\alpha_2\beta\delta_2$ receptors (12, 20, 21). These results provide strong evidence that residues 49 through 61 in the AChR ε subunit form a β strand.

Alternatively, lysine mutations at even numbered positions may produce a subunit that can assemble with complementary subunits, but the mutations reduce the efficiency of receptor assembly. To distinguish between omission of the ε subunit and inefficient assembly, we took advantage of the observation that ligand binding properties of the receptor depend on subunit composition (12, 20, 22). Thus for each mutation, we measured binding of ACh and α-conotoxin GI (CTx GI) by competition against the initial rate of $^{125}$I-α-bgt binding. Receptors containing the ε subunit ($\alpha_2\beta\varepsilon\delta$) show a monophasic competition curve for the agonist ACh, whereas receptors lacking the ε subunit ($\alpha_2\beta\delta_2$) show a distinctive biphasic curve with a plateau at half occupancy extending over three orders of magnitude in ACh concentration (12, 21) (Fig. 1b). Analogous results are obtained with the competitive antagonist CTx GI, where $\alpha_2\beta\varepsilon\delta$ receptors show a competition curve with two closely
spaced components, whereas subunit-omitted receptors show a high affinity component accompanied by a plateau extending over nearly three orders of magnitude of CTx GI concentration. Thus competition profiles for ACh and CTx GI clearly distinguish receptors containing the ε subunit from those with the composition α₂βδ₂.

For the odd numbered lysine mutations, both ACh and CTx GI fully compete against binding of the reporter ligand ¹²⁵I-α-bgt (Fig. 1b). Quantitative differences in the competition profiles are observed among the various mutations because residues in this region contribute to the ligand binding site (23, 24). By contrast, lysine mutations at even numbered positions lead to biphasic profiles characteristic of subunit-omitted α₂βδ₂ receptors (Fig. 1b). Together with the alternating pattern of high and low expression, these results indicate that lysine mutations at odd numbered positions permit incorporation of ε subunits into pentameric AChRs, whereas lysine mutations at even numbered positions lead to ε subunits unable to incorporate into pentamers.

For even-numbered lysine mutations in strand β₂, all but one show a plateau in the competition curve corresponding to approximately 50% of the binding sites, as observed for subunit-omitted α₂βδ₂ receptors. However, for the εI56K mutation, the plateau corresponded to 28% of the binding sites (Fig. 1b). Because a plateau of 50% corresponds to 100% α₂βδ₂ receptors, a plateau of 28% indicates 56% α₂βδ₂ and 44% containing the mutant ε subunit (i.e. α₂βδεI56K). Coexpression of the εI56K mutant yields total expression of α-bgt binding sites of 25% of control, of which only 11% contain the mutant ε subunit. The reduced expression, together with the reduced plateau in the competition measurements, mirrors observations for a disease-causing mutation in the human ε subunit (12). Thus the εI56K mutation markedly impairs the ability of the subunit to oligomerize into assembled receptors.
Residues 49 through 61 are analogous to those in strand $\beta_2$ of AChBP, which is the center of three antiparallel $\beta$ strands that form a sheet delimiting a hydrophobic core on one face and the protein surface on the other (1). The inability of subunits with even numbered lysine mutations to assemble with complementary subunits likely owes to a large energetic penalty for placing a positively charged side chain in a hydrophobic core, together with the hydrogen bonded $\beta$ strands that attempt to hold it in place. We therefore conclude that residues 49 through 61 in the $\epsilon$ subunit form a $\beta$ strand.

Figure 2 exhibits structures of the antiparallel $\beta$ sheet containing strand $\beta_2$ from our model of the $\epsilon$ subunit described herein and the corresponding $\beta$ sheet in AChBP. In strand $\beta_2$ of the $\epsilon$ subunit, residues intolerant to lysine mutation are highlighted in blue, while those tolerant to mutation are highlighted in yellow. In strand $\beta_2$ of AChBP, residues projecting into the hydrophobic core are highlighted in blue, whereas those on the protein surface are highlighted in yellow. Our results demonstrate that residues intolerant to lysine mutagenesis project their side chains into the hydrophobic core, whereas those tolerant to lysine mutagenesis project their side chains on the protein surface. Moreover, the results establish alignment of equivalent residues between the $\epsilon$ subunit and AChBP where key residues contributing to the ligand binding site in the $\epsilon$ subunit, $\epsilon$W55 and $\epsilon$D59, align with their counterparts in AChBP (W53 and T57).

**Lysine scanning mutagenesis of putative $\beta$ strands 1 through 10 of the $\epsilon$ subunit**

We next applied lysine scanning and ligand competition measurements to the nine remaining regions in the $\epsilon$ subunit that potentially form $\beta$ strands. For these measurements, we used a single ligand, CTx GI, to distinguish receptors that incorporate the mutant $\epsilon$ subunit from those that do not (i.e. subunit-omitted $\alpha_2\beta_2\delta_2$ receptors). Lysine mutations that permit incorporation into pentameric AChRs are identified as those yielding $\alpha$-bgt binding sites in the range observed for wild type AChR, together with complete competition by CTx GI against the initial rate of $\alpha$-bgt binding. Lysine mutations that
prevent incorporation into pentameric AChRs are identified as those yielding low expression of $\alpha$-bgt sites, together with the signature biphasic competition profile for CTx GI indicating subunit-omitted receptors.

Out of 110 lysine mutations examined, the majority yielded clear-cut distinction between those that incorporate into pentameric AChRs and those that do not (Table 1). However, nine mutations yielded intermediate results suggesting both assembly-competent and assembly-incompetent $\varepsilon$ subunits. For these mutations, competition profiles for CTx GI were similar to but quantitatively distinct from that for subunit-omitted $\alpha_2\beta\delta_2$ receptors (Fig. 3a). In particular, the plateau in the competition profiles ranged from 5 to 35% of the maximal rate of $\alpha$-bgt binding, rather than the expected 50%, as seen for the mutation $\varepsilon$I56K in strand $\beta2$ (Fig. 1b). Furthermore, close inspection of the competition profiles revealed a component with intermediate affinity for CTx GI, suggesting the presence of receptors containing the mutant $\varepsilon$ subunit along with subunit-omitted $\alpha_2\beta\delta_2$ receptors. In support of this interpretation, the CTx GI competition data are well fitted by the weighted sum of contributions of $\alpha_2\beta\varepsilon\delta$ and $\alpha_2\beta\delta_2$ receptors (Fig. 3a; Table 2).

To confirm that mutant $\varepsilon$ subunits in this intermediate assembly class are present in cell-surface AChRs, we carried out competition measurements using metocurine, the fully methylated analog of $d$-tubocurarine, which binds to the $\alpha$–$\varepsilon$ site with high affinity. The metocurine competition measurements reveal a high affinity component characteristic of the $\alpha$–$\varepsilon$ site, along with a low affinity component characteristic of the $\alpha$–$\delta$ site (Fig. 3b and Table 2). Relative contributions of high and low affinity components, determined from fitting the metocurine competition data, differ among the different mutations, but are similar to the relative contributions estimated from fitting the CTx GI competition measurements (Table 2). Thus competition measurements using metocurine confirm intermediate assembly characteristics of several mutations.
We classified mutations with intermediate assembly characteristics into assembly-competent and assembly-incompetent categories. The assembly-incompetent category comprises εI56K, εL92K, εW118K, εS148K, εF160K and εI215K. These mutations yield similar numbers of α₂βεδ and α₂βδ₂ receptors, while reducing expression of receptors containing the mutant ε subunit to 10 to 15 % compared to the wild type ε subunit (Table 2). These hallmarks of reduced expression parallel those described for a disease-causing mutation in the human ε subunit (12), supporting the classification as assembly-incompetent. The assembly-competent category comprises εP191K, εI194K and εT208K; these incorporate into α₂βεδ receptors, expression is 30 to 50% compared to the wild type ε subunit, and subunit-omitted α₂βδ₂ receptors form.

Four other mutations, εL109K, εY111K, εT117K and εP121K, express in high amounts similar to wild type AChR, yet show biphasic competition curves for CTx GI. Because each of these residues contributes to the ligand binding site (4, 6), the biphasic competition profiles likely owe to impaired binding of CTx GI to the mutant binding sites. To confirm that these mutant ε subunits incorporate into pentamers, rather than yield subunit-omitted receptors, we examined ACh competition against ¹²⁵I-α-bgt binding. The mutations εY111K and εT117K exhibit monophasic competition profiles similar to wild type AChR, confirming that these mutant subunits incorporate into cell-surface AChRs (Fig. 3c). The mutations εL109K and εP121K show broad competition profiles shifted to high concentrations of ACh, but the profiles clearly differ from that for subunit-omitted α₂βδ₂ receptors (compare Figs. 1b and 3c). Thus although these four mutations impair ligand binding, they incorporate into cell-surface AChRs. In summary, out of 110 mutations examined, 68 permit incorporation into cell-surface receptors, whereas 42 produce subunits with limited or no ability to incorporate into surface receptors.
In addition to experimental results for 110 mutations, we infer surface or core side chain dispositions for 12 more residues. Residues with positively charged side chains are assumed to project on the protein surface; these include εK34, εR79, εR125, εR147, εR195, εR196, εR202 and εR203. A glycosylation site, εN141, and its consensus residue, εS143, are also assumed to project on the protein surface. On the other hand, dispositions of the disulfide-bonded cysteines 128 and 142 could not be assessed by mutagenesis, owing to their absolute requirement for subunit folding (25, 26). These cysteines are assumed to project into the hydrophobic core of the subunit, as observed for the corresponding cysteines in AChBP (1).

Homology modeling of the major extracellular domain of the human muscle AChR-

The most important requirement in homology modeling is correct alignment of the sequence to be modeled with that of the template structure. Our determination of side chain disposition in the ε subunit, together with known side chain dispositions in AChBP, allows correct alignment of sequences in the ten β strands and several linkers between the strands. Furthermore, the alignment of ε subunit and AChBP sequences provides a basis for aligning α, β and δ sequences with that of AChBP. Additionally, for five of the ten loops between β strands, alignment is straightforward, as there are no insertions between AChR subunits and AChBP. The remaining five loops contain insertions, and therefore constitute the greatest potential sources of error in modeling; however four of these loops contain one to three insertions, which the homology modeling program MODELER is designed to accommodate (15, 17). The remaining loop is located between strands β8 and β9, and contains an eight to eleven residue insertion in β, ε and δ subunits. Our determinations of side chain disposition are overlaid upon the overall sequence alignment used in modeling (Fig. 4).

We used MODELER in the Insight II software environment to generate a homology model of the major extracellular domain of the adult human AChR using spatial constraints provided by AChBP.
(see Experimental Procedures). To maintain complementarity between subunits at their interfaces, all five subunits were modeled simultaneously. The output structure from MODELER was corrected for misfolded linkers between strands β8 and β9 in β, ε and δ subunits, followed by energy minimization (see Experimental Procedures).

**Mapping the lysine scanning data on the model of the AChR ε subunit**-

The overall mutagenesis results are mapped on our structural model of the ε subunit, using yellow to indicate mutations producing folded and blue to indicate mutations producing misfolded subunits (Fig. 4). The map reveals alternating patterns of folded and misfolded subunits in nine of the ten β strands, while strand β5, which contains two residues, shows no alternating pattern (Fig. 4; Table 1). Nine of the β strands align as pickets of a fence along the vertical axis of the subunit, forming a β sheet that wraps in a cylinder to delimit a hydrophobic core on one face and the protein surface on the other. In the upper half of the sheet, the mutagenesis map reveals precisely aligned stripes of yellow alternating with blue that run normal to the long axis of the subunit. Each row of the yellow or blue encoded residues forms a ring perpendicular to the long axis of the subunit, and these rings stack one upon another.

In the lower portion of the β sheet, the map reveals alternating core and surface side chains, as observed in the upper portion of the sheet, but in several strands consecutive residues project their side chains on the protein surface. These consecutive surface residues correspond to local regions where the strand twists away from the hydrophobic core, exposing consecutive side chains on the protein surface. These twisted regions of strands β1, β6 and β9 correspond to regions in AChBP in which the β strands also twist away from the core (1). Thus lysine scanning not only establishes alignment of side chains between the ε subunit and AChBP, but also identifies local regions where the β strand twists away from the hydrophobic core.
In a departure from the AChBP structure, we find that the side chain of εL40 projects on the protein surface, contrary to the equivalent residue I38 in AChBP, which projects into the hydrophobic core (Fig. 4). Incorporated into our structural model, the surface disposition of εL40 means that five consecutive residues of strand β1 project their side chains on the protein surface. Moreover, surface disposition of εL40 eliminates an inward kink in strand β1 present in AChBP; instead the main chain of strand β1 aligns co-linearly with the main chain of strand β6 as the pair wrap around the ε subunit (Fig. 5). Thus surface exposure of these five consecutive side chains owes to twisting of strand β1 away from the hydrophobic core together with removal of the kink at position ε40. This difference between the AChR ε subunit and AChBP may be structurally important because leucine is conserved at this position in all heteromeric AChR subunits, whereas isoleucine is present in homomeric AChR subunits.

Projection of side chains into the hydrophobic core and on the protein surface-

Space filling rendering shows that residues tolerant to lysine mutagenesis project on the protein surface, whereas residues intolerant to mutagenesis project into the protein core (Fig. 6). To illustrate side chain disposition, we divide the ε subunit into three regions bounding the hydrophobic core: upper, lower and outer regions.

The upper region contributes to the subunit interface formed with the α subunit to create one of two ACh binding sites, and comprises strands β3, β5’, β8, and the upper halves of strands β6, β2 and β1 (Fig. 6a). All residues tolerant to lysine mutagenesis project on the protein surface. Out of 18 side chains that project on the surface, 9 are hydrophobic, including T, L, P and W, while 9 are hydrophilic or charged, including D, N, E, Q, K and R. Residues previously shown to contribute to the ligand binding site, εW55 (24), εL109 (36), εY111 (27), εT117 (13) and εL119 (28), project on the protein
surface. By contrast, all residues intolerant to lysine mutagenesis project into the hydrophobic core. These core residues are exclusively hydrophobic, and include V, L, I, F and W.

The lower region comprises the lower portions of strands $\beta_1$, $\beta_2$, $\beta_6$ and the short $\beta_5$. These collectively form the inner wall of the subunit facing the central vestibule which ions pass in route to the transmembrane channel (Fig. 6b). A relatively large proportion of residues in this region tolerate mutation to lysine, owing to twisting of strands $\beta_1$, $\beta_6$ and $\beta_5$ away from the hydrophobic core. As a result, one edge of the protein backbone projects toward the central vestibule, allowing exposure of polar groups in the backbone and consecutive side chains to the aqueous exterior. Surface exposure of the polar backbone may compensate for the relatively small proportion of hydrophilic side chains on the protein surface. Only 6 out of 18 surface exposed residues in this region are hydrophilic, including S, G, N and R while the remaining residues are hydrophobic, including A, T, V, L, I and Y.

The outer region contributes to the outer wall of the subunit and the subunit interface not involved in ligand binding, and includes strands $\beta_9$, $\beta_10$, $\beta_7$ and $\beta_4$ (Fig. 6c). Residues that tolerate lysine mutagenesis project their side chains on the protein surface, with one exception: $\varepsilon I_{215}$. $\varepsilon I_{215}$ is expected to project on the protein surface, yet is classified as assembly-incompetent because mutation to lysine leads to similar numbers of $\alpha_2\beta\varepsilon\delta$ and $\alpha_2\beta\delta_2$ pentamers and expression is markedly reduced (Table 2). Reduced expression of $\varepsilon I_{215}K$ may owe to instability between the engineered lysine side chain and the endogenous $\varepsilon R_{217}$, which projects on the same side of strand $\beta_{10}$. Of the remaining 15 surface exposed side chains in the outer region, 8 are charged or hydrophilic, similar to the upper hydrophobic region, and include S, D, N, E and R. The remaining surface exposed side chains are hydrophobic and include A, V, I, P and F. All residues intolerant to lysine mutagenesis project their side chains into the hydrophobic core; these are predominantly hydrophobic, including V, L, I, F, Y and W, with S and G the hydrophilic exceptions.
**AChR subunit interfaces**

Our model of the AChR ligand binding domain shows complementarity between juxtaposed subunits, with the characteristic twisting of each subunit forming protrusions and grooves that interlock the pentameric array (Fig. 7). Each pair of subunits shows three regions of close approach at the top, middle and bottom of the interface. Particular residues in each region span the subunit interface, with the partners approaching close enough to interact through a variety of non-covalent forces, including salt bridges, hydrogen bonds and hydrophobicity.

In the top region of inter-subunit contact, our model reveals a salt bridge between αR20 and εE4, and between equivalent residues at each subunit interface (Fig. 7b). These salt bridges are distinct from the bifurcated salt bridge in AChBP (1), which forms between side chains from different regions of the juxtaposed subunits. In further support of a salt bridge at each AChR subunit interface, arginine is present at the aligned position 20 in all species of muscle and neuronal AChR subunits, with Torpedo γ as the lone exception. Similarly, glutamic acid is present at the aligned position 4 in all species of muscle and heteromeric neuronal AChR subunits. These salt bridges may stabilize the pentamer in either open or closed states, and are candidates for breaking and reforming during channel gating, as established for salt bridges in prototypical allosteric proteins (29).

The middle region of inter-subunit contact gives rise to the ACh binding sites, which are accessible from the periphery of the pentamer (Fig. 7 b, c). These are formed at α–ε and α–δ subunit interfaces, as established by mutagenesis, site-directed labeling and the AChBP structure. The center of the ACh binding pocket contains aromatic and hydrophobic side chains from each interface partner. The aromatic residues, αW149, αY93 and εW55, merge at the center of the binding site, and these are flanked by the hydrophobic residues αV91, εL119, εP121 and εT36 (Fig. 7c). Additional aromatic residues, αY190 and αY198, line the outer wall of the binding site cavity. Each of these nine residues,
or their aligned equivalents, is highly conserved and present at both $\alpha-\varepsilon$ and $\alpha-\delta$ binding sites; they may constitute minimal structures necessary for ACh binding. Residues $\varepsilon L109$, $\varepsilon T117$ and $\varepsilon D59$ form a row above and to the right of the binding site. $\varepsilon D59$, which is not conserved, contributes to selectivity of competitive antagonists for the $\alpha-\varepsilon$ over the $\alpha-\delta$ site, because it differs from its counterpart in the $\delta$ subunit, $\delta G61$ (23). Similarly $\gamma Y117$, and its equivalent residue $\delta T119$ in the fetal mouse AChR, contribute to selectivity of competitive antagonists for the $\alpha-\gamma$ over the $\alpha-\delta$ site (13). These and other non-conserved residues at the binding site may constitute structures that fine tune ACh binding to meet the demands of the motor synapse in a given species.

The bottom region of inter-subunit contact contains residue partners that may form hydrogen bonds across the interface (Fig. 7b). At the $\alpha-\varepsilon$ site, these residues include $\alpha Y127$, $\varepsilon N39$ and $\varepsilon N182$, while equivalent residues are present at the $\alpha-\delta$ site. Residue $\alpha Y127$ flanks $\alpha C128$ that forms the signature cys-loop found in all AChR subunits, $\varepsilon N39$ projects on the protein surface from strand $\beta 1$, and $\varepsilon N182$ is located in the linker just amino-terminal to strand $\beta 9$. Residues $\alpha Y127$ and $\varepsilon N39$ are situated close enough to form an amide-aromatic hydrogen bond. Toward the periphery from this pair, the amide group of $\varepsilon N182$ closely approaches the main chain carbonyl of $\alpha C128$ to which it may form a hydrogen bond. $\alpha Y127$, $\varepsilon N39$ and $\varepsilon N182$ are conserved in all species of the corresponding AChR subunits, suggesting critical contributions at both ligand binding interfaces.
Discussion-

We introduce lysine scanning mutagenesis as a method to delineate repeating structures that separate hydrophobic from hydrophilic regions in proteins. The method takes advantage of instability of the positively charged side chain in a hydrophobic environment, and of its stability in the polar environment of the protein surface. We show that the AChR ε subunit contains β-strands that delimit a hydrophobic core, and compare predicted side chain dispositions with known dispositions in the homologous AChBP (1). Our results establish register of side chains between β strands in the ε subunit and those in AChBP, identify local twisted regions in the β-strands and identify structural differences between AChBP and the AChR. Our experimentally determined sequence alignment provides the basis for building a homology model using spatial constraints from the homomeric AChBP. The resulting structural model provides a detailed picture of the ACh binding site of the adult human AChR, which is heteromeric and contains multiple conserved residues that differ from residues in equivalent positions in AChBP. The model also discloses previously unrecognized residue pairs at subunit interfaces, which emerge as candidates for mediating allostERIC transitions triggered by agonist. The structural model provides clear predictions that can be tested by mutagenesis to further refine the structure and provide insight into function. The overall findings suggest that lysine scanning may be used to generate reliable structural models of other members of the AChR superfamily, as well of other proteins not amenable to x-ray crystallography or NMR.

Lysine is rarely found in protein interiors unless stabilized by other charged or polar groups. An engineered lysine is not likely to encounter such stabilizing groups in the protein interior, and therefore should be highly unstable. When the engineered lysine is located in a repeating structure such as a β-strand, main chain hydrogen bonds attempt to hold it in place, causing a structural perturbation that prevents oligomerization. Lysine scanning mutagenesis has been applied to
peripheral loops in human thyrotropin to produce superactive analogs (38), and to voltage-gated sodium channels to identify sites for local anesthetic block (39). However, the present study is the first to apply lysine scanning to distinguish core hydrophobic from surface hydrophilic environments of residue side chains. Using ligands specific for subunit composition, our experiments assess the presence or absence of the structural perturbation by determining whether the mutant subunit oligomerizes with complementary wild type subunits to form cell-surface AChRs. Null mutants, which do not oligomerize, give rise to subunit-omitted $\alpha_2\beta\delta_2$ pentamers, which produce a unique biphasic ligand binding signature (12, 21). The two ligand binding sites in the $\alpha_2\beta\delta_2$ pentamer bind $\alpha$-bgt at the same rate, but only one of these binds ACh and CTx GI with high affinity. Why only one binding site in $\alpha_2\beta\delta_2$ receptors achieves high affinity for these ligands is not known, but owes to the absence of the $\epsilon$ subunit, which allows ready identification of null mutants.

The null mutants likely fold partially and associate with $\alpha$ and $\beta$ subunits, but these do not assemble into cell-surface AChRs. Evidence for partial folding and association comes from our observation that the number of $\alpha$-bgt binding sites was reduced in the presence of the null mutants compared to $\alpha$, $\beta$ and $\delta$ subunits alone (Table 1; Fig. 1a); the null mutant $\epsilon$ subunits likely compete for $\alpha$ and $\beta$ subunits, reducing expression of $\epsilon$-omitted receptors. Out of 110 mutants examined, nine exhibit detectable but incomplete incorporation into cell surface AChRs, as both $\epsilon$-containing and $\epsilon$-omitted receptors are detected. All but one of these mutations localizes to the C-terminus of a $\beta$-strand (Fig. 4; Table 2), a position that may more readily allow the strand to twist away from the hydrophobic core.

Our structural model of the AChR extracellular domain (Fig. 7) shows differences as well as similarities compared to the AChBP structure (1). Because our experimental results allowed side chain alignment in the core $\beta$ strands, these core regions are the most similar between the two proteins.
However, strand β1 shows altered side chain disposition in a stretch of consecutive surface exposed residues, which we incorporate into our structural model (Fig. 5). Additional differences are evident in the peripheral strands β9 and β10; these are greatest in non-α subunits, which contain one or more prolines in strand β9, unlike AChBP, and smallest in the α subunit, which lacks proline in this region (Fig. 4). Differences are also present in the ligand binding sites where residues conserved in all heteromeric AChRs, εT36, αY91, εL109, εL119, and εP121, differ from the corresponding aligned residues in the homomeric AChBP, K34, A87, R104, M114 and S116 (Figs. 4 and 7). Larger differences are evident in linkers between β-strands, particularly the linkers between strands β8 and β9 and between strands β9 and β10. For the β8-β9 linker, the differences are small in the α subunit which contains only one residue inserted, but largest in the non-α subunits, which contain eight to eleven inserted residues. For the β9-β10 linker, differences are again smallest in the α subunit, which contains a single inserted residue and the vicinal cysteines 192 and 193, also present in AChBP, and greatest in non-α subunits, which contain one to three inserted residues and lack vicinal cysteines.

The ACh binding sites are formed at α–ε and α–δ subunit interfaces, as established by mutagenesis, site-directed labeling and the AChBP structure. Mutagenesis and site-directed labeling established that seven loops, far apart in the linear sequence, contribute to the ACh binding site: loops A, B and C in the α subunit and loops D, E, F and G in ε or δ subunits (4, 6). The AChBP structure confirmed that residues in each of these loops are present at the ACh binding site, and our model provides a detailed picture of both common and unique residues in the human muscle AChR. In the AChR, residues in each of the seven loops localize to the ligand binding site: αY93 in loop A, αW149 in loop B, αY190 and αY198 in loop C, εT36 in loop D, εW55 in loop E, εL119 and εP121 in loop F and εD175 in loop G. Each of these residues is highly conserved and may constitute minimal structures necessary for ACh binding. Additional residues, predominantly non-conserved, have been
identified by mutagenesis and are located at the periphery of the binding site. These include αG153 in loop B (37), αS187, αV188 and αT189 in loop C (18, 30), εK34 in loop D (27, 31), εD59 in loop E (23), εL109, εY111, εS115 and εT117 in loop F (13, 27, 32, 36) and εE177 in loop G (18, 33). These peripheral residues may constitute structures specialized for binding ACh at concentrations found at the motor synapse, or for releasing bound ACh with sufficient speed to terminate the response.

Beyond their importance in forming the ACh binding sites, subunit interfaces are critical because the fundamental twisting movement in channel activation displaces one subunit relative to its neighbors (11). Inter-subunit interactions can be broadly classified into those stabilizing resting, active or desensitized states, and further classified into strong or weak interactions. The strong interactions are candidates for breaking and reforming during allosteric transitions between functional states, whereas the weak interactions may provide complementary surfaces between juxtaposed subunits in one state or the other. Our model contains a salt bridge between αR20 and εE4, as well as between equivalent residues at each subunit interface (Fig. 7b). The salt bridges in the AChR are distinct from the bifurcated salt bridges in AChBP (1), which form between side chains from different regions of the juxtaposed subunits. Salt bridges in the AChR may provide strong inter-subunit stabilization in one functional state or another, and therefore emerge as candidates for breaking and reforming during channel gating or desensitization, analogous to salt bridges that break and reform during activation of classical allosteric proteins (29). Thus our results provide tangible starting points for future experiments aimed at correlating AChR structure with function.

A general theme regarding subunit interface structure emerges from our model of the AChR, namely that each subunit interface contains contact regions that are essentially the same at all interfaces, as well as contact regions that are unique and highly specialized. Common interface structures include the salt bridges at the top region of contact in all subunits. Specialized structures
include the ligand binding sites, which are present only at \( \alpha - \varepsilon \) and \( \alpha - \delta \) interfaces. At the remaining three subunit interfaces analogous to the ACh binding site, specialized contact residues are present and likely make critical contributions to function. For example at the \( \varepsilon - \alpha \) interface, \( \varepsilon E93 \) (equivalent to \( \alpha Y93 \)) closely approaches \( \alpha R55 \) (equivalent to \( \varepsilon W55 \)), suggesting a salt bridge. \( \alpha R55 \) is conserved in all muscle \( \alpha \) subunits, while \( \varepsilon E93 \) is conserved in all \( \varepsilon \) and \( \gamma \) subunits, supporting a critical functional role for a salt bridge between these residues.

Our model of the AChR extracellular domain naturally has limitations. Regions containing insertions relative to AChBP are the greatest sources of uncertainty in modeling; these comprise five of the ten linkers between \( \beta \)-strands and the peripheral \( \beta \)-strands \( \beta 9 \) and \( \beta 10 \). The linker between strands \( \beta 8 \) and \( \beta 9 \) requires further modeling, perhaps using distance constraints between bound ligand and residue side chains, combined with measurements of ligand binding following mutation of candidate residues. The three \( \alpha \)-helices predicted from AChBP have not been examined in the AChR, but as they lodge against the hydrophobic core, they could be examined by lysine scanning. Our results from mutating \( \varepsilon I215 \) is not readily explained by our structural model, as the \( \varepsilon T215K \) mutation incorporates only partially into cell-surface receptors, yet its side chain is predicted to project onto the protein surface. A likely explanation is the \( \varepsilon T215K \) mutation places its positive charge proximal to the endogenous residue \( \varepsilon R217 \) on the same side of the \( \beta \) strand, which is unstable due to electrostatic repulsion.

Lysine scanning may also prove useful when applied to other structurally intractable proteins. It may allow side chain disposition to be determined in other regions of the AChR \( \varepsilon \) subunit, including helices and perhaps short linkers. Longer linkers are expected to be flexible and allow reorientation of the substituted lysine side chain, unlike \( \beta \)-sheets where hydrogen bonds hold the main chains in place and thus constrain the side chains. Lysine scanning also holds promise for examining the AChR \( \alpha, \beta \)
and δ subunits, as well as subunits in other members of the AChR superfamily, such as GABA<sub>A</sub>, glycine and 5-HT-3 receptors. Perhaps the greatest potential lies in deducing reliable structural models of the many intractable proteins expected to emerge from genomics and proteomics studies. When an unknown protein is homologous to a known protein with repeating structures delimiting a hydrophobic core, secondary structure and side chain register can be determined by lysine scanning coupled with a measure of protein folding. The results should allow correct alignment of model and template sequences, and generation of reliable homology models.

Future studies will be aimed at refining our structural model of the AChR ligand binding domain. These refinements will likely require distance constraints generated by docking ligands whose three-dimensional structures are known, coupled with the effects of mutations on their binding. Molecular dynamics simulations are possible with the structural model presented here, and will yield additional refinement by including explicit solvent molecules to provide more realistic energy minimization of the structure. Once a refined structural model is obtained, simulation of ligand association with the ACh binding site should reveal the nature of molecular recognition in the AChR.
Figure legends-

Figure 1- Lysine scanning mutagenesis of residues 49-61 of the ε subunit. (a) Fractional expression in 293 HEK cells transfected with α, β, δ and the indicated ε subunits is expressed as the number of cell-surface $^{125}$I-α-bgt binding sites relative to wild type α₂βεδ AChRs (see Experimental Procedures). (b) Competition of ACh and α-CTx GI against the initial rate of $^{125}$I-α-bgt binding for control receptors, α₂βεδ and α₂βδ₂, and the indicated lysine mutations. For the ACh competition measurements, the curves were obtained by fitting either the Hill equation (Eq. 1, see Experimental Procedures) or the two-site equation (Eq. 2), with the fitted parameters given in Table 3. For the CTx GI competition measurements, the smooth curves are fits using the two-site equation (Eq. 2) or one site plus a constant (Eq. 3), with the fitted parameters given in Table 1; the fitted curve for I56K was obtained using Eq. 4, with the fitted parameters given in Table 2. Mutations are indicated as follows: open squares: T49K and L50K; filled squares: T51K and T52K; open circles: S53K and V54K; filled circles: W55K and I56K.

Figure 2- Mapping side chain dispositions from lysine scanning on our model of the AChR ε subunit and the known dispositions in AChBP (1). The antiparallel strands β1, β2 and β6 are shown, with space-filling rendering of residues in strand β2; residues colored yellow project on the protein surface, whereas those colored blue project into the hydrophobic core.

Figure 3- Ligand competition analysis for lysine mutations with atypical expression properties. (a) CTx GI competition against the initial rate of $^{125}$I-α-bgt binding to cells transfected with the mutations εS148K and εP191K together with complementary wild type subunits. For reference, the thin lines are mean results for α₂βεδ and α₂βδ₂ receptors obtained by fitting Eqs. 2 and 3, respectively. Smooth curves through the data are fits to the weighted sum of these two equations (Eq. 5), assuming 50% high affinity sites for α₂βδ₂ receptors, with parameters given in Table 2.
(b) Metocurine competition measurements for the indicated mutations and control receptors. The smooth curves are fits obtained using the two-site equation in which the fraction of each site is variable (Eq. 4); fitted parameters are given in Table 2. (c) ACh competition measurements for the indicated receptors. The smooth curves are fits obtained using either Eq. 1 or Eq. 2, with fitted parameters given in Table 3.

**Figure 4**- Side chain dispositions and resulting sequence alignments of AChR subunits with AChBP (*lower panel*). Surface and core residues are highlighted in yellow or blue, respectively, for the experimentally determined orientations from lysine scanning the AChR ε subunit and AChBP (1). For AChBP, surface and core side chain orientations were assessed from side chain contacts using the program 3D-PSSM (35), and confirmed by visual inspection of the structure. β-strands are shown above the sequences. *Upper panel* shows secondary structure rendering of the AChR ε subunit, with results from lysine scanning overlaid. Regions colored *yellow* correspond to positions where lysine mutation produced a folded subunit, whereas regions colored *blue* correspond to positions where lysine mutation produced a misfolded subunit. *Left* view is from the subunit interface that forms the ligand binding site with the α subunit. *Right* view is rotated 180°, and shows the opposite interface and the region facing the inner vestibule.

**Figure 5**- Comparison of side chain dispositions of εL40 and AChBP I38 and local structures of the main chains of strand β1. Strands of the ε subunit are colored *yellow* and AChBP colored *green*. Note εL40 projects on the protein surface and allows smooth bending of the main chain and co-alignment of strands β1 and β2, whereas I38 projects into the hydrophobic core and coincides with a kink in the main chain of strand β1.

**Figure 6**- Space filling rendering of residues in three regions of the AChR ε subunit. Results from lysine scanning are shown, with residues in *yellow* corresponding to folded and residues in
blue corresponding to misfolded following mutation to lysine. (a) β-sheet delimiting the upper hydrophobic core, including strands β3, β5’, β8 and the upper halves of β6 and β1. Left view is from the subunit interface that forms the ligand binding site with the α subunit, and right view is rotated 180°. (b) β-sheet facing the inner hydrophobic core, including strands β5 and the lower portions of β1, β2 and β6. Left view is from the inner vestibule, and right view is from the top and rotated 180°. (c) β-sheet delimiting the outer hydrophobic core, including strands β9, β10, β7 and β4. Left view is from the outer surface of the subunit, and right view is from the top of the subunit.

**Figure 7**- Homology model of the overall structure of the ligand binding domain of the adult human AChR. Panel a views the structure from the top with the α subunit highlighted in magenta, the ε subunit in yellow, and the remaining three subunits in gray. Panel b shows the structure from the side with residue side chains highlighted at three key regions of close approach of the subunits (see text). Panel c shows a stereo view of the ACh binding site at the α–ε subunit interface. Main chains are magenta for the α subunit and yellow for the ε subunit. Side chains of key conserved aromatic and hydrophobic residues are displayed, along with three non-conserved residues that align in a row above and to the right of the binding site center (see text).
Table 1 - Expression and CTx GI Competition Parameters for Lysine mutations.

| Strand | β1 | β2 | β3 | β4 | β5 |
|--------|----|----|----|----|----|
| AChR ε | AChBP | Expression | Ka | Kb | Fraction a |
| αεβεδ | - | 1.0 | 1.9E-9 | 1.1E-7 | 0.5 |
| αεβδε | - | 0.3 | 5.8e-9 | NC | 0.52 |
| T28K | P26 | 0.68 | 2.7E-9 | 5.2E-8 | 0.5 |
| V29K | V27 | 0.22 | 4.2E-9 | NC | 0.55 |
| T30K | A28 | 1.1 | 2.2E-9 | 8.7E-8 | 0.5 |
| I31K | V29 | 0.19 | 5.3E-9 | NC | 0.55 |
| S32K | S30 | 1.08 | 1.3E-8 | 2.6E-8 | 0.5 |
| L33K | V31 | 0.19 | 6.8E-9 | NC | 0.57 |
| K34 | S32 | NM | | | |
| V35K | L33 | 0.19 | 8.1E-9 | NC | 0.59 |
| T36K | K34 | 0.58 | 1.8E-9 | 1.7E-8 | 0.5 |
| L37K | F35 | 0.17 | 3.8E-9 | NC | 0.54 |
| T38K | I36 | 1.19 | 1.1E-9 | 1.2E-8 | 0.5 |
| N39K | N37 | 0.42 | 2.6E-9 | 5.6E-9 | 0.5 |
| L40K | I38 | 0.61 | 2.4E-9 | 3.2E-8 | 0.5 |
| I41K | L39 | 0.7 | 1.1E-9 | 1.6E-8 | 0.5 |
| S42K | E40 | 1 | 1.6E-9 | 3.5E-8 | 0.5 |
| L43K | V41 | 0.39 | 3.3E-9 | NC | 0.56 |
| N44K | N42 | 0.91 | 2.3E-9 | 1.5E-7 | 0.5 |
| T49K | E47 | 0.92 | 1.0E-9 | 5.8E-8 | 0.5 |
| L50K | V48 | 0.18 | 3.2E-9 | NC | 0.58 |
| T51K | D49 | 0.79 | 3.9E-9 | 3.6E-8 | 0.5 |
| T52K | V50 | 0.17 | 3.6E-9 | NC | 0.49 |
| S53K | V51 | 0.69 | 1.3E-9 | 1.4E-8 | 0.5 |
| V54K | F52 | 0.19 | 3.2E-9 | NC | 0.48 |
| W55K | W53 | 0.66 | 2.3E-9 | 1.3E-8 | 0.5 |
| I56K | Q54 | 0.25 | 2.0E-8 | NC | 0.7 |
| G57K | Q55 | 0.69 | 8.3E-9 | 8.3E-9 | 0.5 |
| I58K | T56 | 0.21 | 1.8E-8 | NC | 0.62 |
| D59K | T57 | 0.77 | 1.4E-9 | 2.3E-7 | 0.5 |
| W60K | W58 | 0.15 | 3.4E-9 | NC | 0.58 |
| Q61K | S59 | 1.01 | 7.70E-10 | 8.1E-8 | 0.5 |
| I75K | P71 | 0.27 | 3.0E-9 | NC | 0.51 |
| E76K | D72 | 0.83 | 2.0E-9 | 2.0E-7 | 0.5 |
| T77K | Q73 | 0.81 | 4.0E-9 | 6.3E-7 | 0.5 |
| L78K | V74 | 0.23 | 6.0E-9 | NC | 0.53 |
| R79 | S75 | NM | | | |
| V80K | V76 | 0.22 | 7.9E-9 | NC | 0.43 |
| I90K | L86 | 0.22 | 1.7E-9 | NC | 0.48 |
| V91K | A87 | 0.7 | 5.6E-9 | 9.3E-9 | 0.5 |
| L92K | A88 | 0.37 | 1.3E-8 | NC | 0.79 |
| E93K | Y89 | 0.79 | 4.1E-9 | 1.1E-8 | 0.5 |
| G101K | E96 | 0.98 | 3.0E-9 | 2.0E-7 | 5.0E-1 |
| Strand | β 5−5' linker | β 5−6 linker | Strand | β 6−7 linker | Strand | β 7−8 linker | β 8−9 linker |
|--------|---------------|---------------|--------|---------------|--------|---------------|---------------|
|        | V102K         | V97           | 1      | 4.1E-9        | 3.5E-7 | 5.0E-1        |                |
| β 5−5' | A103K         | L98           | 1.01   | 3.6E-9        | 1.99E-07 | 5.0E-1        |                |
|        | Y104K         | T99           | 0.95   | 1.5E-9        | 1.9E-8 | 5.0E-1        |                |
|        | D105K         | P100          | 0.89   | 2.5E-9        | 9.9E-8 | 0.5           |                |
|        | A106K         | Q101          | 0.39   | 1.5E-9        | 1.3E-8 | 0.5           |                |
| N107K  | L102          | 0.81          | 3.3E-9 | 2.8E-8        | 0.5    |                |                |
| V108K  | A103          | 0.2           | 8.1E-9 | NC            | 0.57   |                |                |
|        | L109K         | R104          | 0.77   | 3.7E-9        | NC     | 0.7           |                |
| V110K  | V105          | 0.18          | 6.4E-9 | NC            | 0.49   |                |                |
|        | Y111K         | V106          | 0.79   | 3.1E-9        | NC     | 0.62          |                |
| E112K  | S017          | 0.75          | 1.1E-9 | 2.6E-8        | 0.5    |                |                |
| G113K  | D108          | 0.67          | 1.1E-9 | 5.3E-8        | 0.5    |                |                |
| G114K  | G109          | 0.12          | 4.9E-9 | NC            | 0.52   |                |                |
| S115K  | E110          | 0.97          | 1.4E-9 | 7.6E-8        | 0.5    |                |                |
| V116K  | V111          | 0.09          | 5.0E-9 | NC            | 0.55   |                |                |
| T117K  | L112          | 0.94          | 3.5E-9 | NC            | 0.59   |                |                |
| W118K  | Y113          | 0.17          | 6.4E-9 | NC            | 0.71   |                |                |
| L119K  | M114          | 0.48          | 3.8E-9 | 3.8E-9        | 0.5    |                |                |
| P120K  | P115          | 0.95          | 1.5E-9 | 1.4E-8        | 0.5    |                |                |
| P121K  | S116          | 1.05          | 4.7E-9 | NC            | 0.5    |                |                |
| A122K  | I117          | 1.11          | 1.4E-9 | 1.95E-8       | 0.5    |                |                |
| I123K  | R118          | 0.72          | 9.00E-10 | 2.1E-8  | 0.5    |                |                |
| Y124K  | Q119          | 0.54          | 8.60E-10 | 1.5E-8  | 0.5    |                |                |
| R125   | R120          | NM            |        |                |        |                |                |
| S126K  | F121          | 0.18          | 1.9E-8 | NC            | 0.62   |                |                |
| V127K  | S122          | 0.9           | 1.4E-9 | 2.1E-8        | 0.5    |                |                |
| C128   | C123          | NM            |        |                |        |                |                |
| N141   | T135          | NM            |        |                |        |                |                |
| C142   | C136          | NM            |        |                |        |                |                |
| S143   | R137          | NM            |        |                |        |                |                |
| L144K  | I138          | 0.18          | 1.2E-8 | NC            | 0.52   |                |                |
| I145K  | K139          | 0.65          | 3.2E-9 | 1.4E-8        | 0.5    |                |                |
| F146K  | I140          | 0.17          | 3.0E-9 | NC            | 0.43   |                |                |
| R147   | G141          | NM            |        |                |        |                |                |
| S148K  | S142          | 0.26          | 1.2E-8 | NC            | 0.66   |                |                |
| E155K  | E149          | 0.51          | 1.4E-9 | 1.8E-8        | 0.5    |                |                |
| V156K  | I150          | 0.22          | 1.2E-8 | NC            | 0.54   |                |                |
| E157K  | S151          | 0.95          | 2.7E-9 | 2.4E-7        | 0.5    |                |                |
| F158K  | V152          | 0.2           | 7.0E-9 | NC            | 0.44   |                |                |
| T159K  | D153          | 1.04          | 2.6E-9 | 1.8E-8        | 0.5    |                |                |
| F160K  | P154          | 0.29          | 1.5E-8 | NC            | 0.68   |                |                |
| A161K  | T155          | 0.28          | 6.5E-9 | NC            | 0.55   |                |                |
| V162K  | T156          | 1             | 2.7E-9 | 2.2E-7        | 0.5    |                |                |
| D163K  | E157          | 0.63          | 4.5E-9 | 4.5E-5        | 0.81   |                |                |
| Strand β9 |  |  |  |  |
|---|---|---|---|---|
| E184K | R170 | 0.81 | 1.9E-9 | 2.2E-8 | 0.5 |
| W185K | F171 | 0.23 | 5.4E-9 | NC | 0.51 |
| A186K | E172 | 1 | 2.9E-9 | 2.6E-8 | 0.5 |
| I187K | I173 | 0.25 | 7.6E-9 | NC | 0.51 |
| D188K | L174 | 0.77 | 2.1E-9 | 2.4E-8 | 0.5 |
| F189K | D175 | 0.71 | 1.5E-9 | 2.2E-8 | 0.5 |
| C190K | V176 | 0.23 | 9.0E-9 | NC | 0.58 |
| P191K | T177 | 0.52 | 7.8E-9 | 1.2E-5 | 0.68 |
| G192K | Q178 | 0.27 | 2.3E-9 | NC | 0.5 |
| V193K | K179 | 1.03 | 2.8E-9 | 1.3E-7 | 0.5 |
| I194K | K180 | 0.39 | 2.3E-8 | NC | 0.89 |
| R195 | N181 | NM |  |  |  |

Expression is the total number of cell-surface α-bgt binding sites relative to that for wild type α2βεδ AChRs (see Fig. 1a and Experimental Procedures). For receptors in which CTx GI competed against all α-bgt binding (Fig. 1b), Ka and Kb are dissociation constants determined by fitting Eq. 2 to the data. For receptors in which Ctx GI did not compete against all α-bgt binding (highlighted in bold; see Fig. 1b), Ka is the dissociation for the high affinity component, and Fraction a is the fraction of sites with dissociation constant Ka determined by fitting Eq. 3 to the data; NC indicates that a second component was present but that there was
no competition at CTx GI concentrations greater than 30 µM (see Fig. 1b). For AChBP, residues highlighted in bold orient toward the hydrophobic core (see Fig. 4 legend). NM indicates residue was not mutated.
Table 2- Ctx GI and Metocurine competition parameters for lysine mutations yielding subunit-containing (α2βεδ) and subunit-omitted receptors (α2βδ2).

| Mutant | Ctx GI Parameters | Metocurine Parameters |
|--------|-------------------|-----------------------|
|        | $K_a$ | $K_b$ | Fraction two-site | $\alpha_2\beta\delta_2/\alpha_2\beta\epsilon\delta$ | $K_a$ | $K_b$ | Fraction $K_a$ | $\alpha_2\beta\delta_2/\alpha_2\beta\epsilon\delta$ |
| $\alpha_2\beta\epsilon\delta$ | 1.9E-9 | 1.1E-7 | 1.0 | 0.0 | 7.2E-8 | 6.0E-6 | 0.5 | 0.0 |
| I56K   | 7.4E-9 | 5.7E-7 | 0.54 | 0.85 | 1.5E-7 | 9.5E-6 | 0.25 | 1.0 |
| L92K   | 4.1E-9 | 3.1E-7 | 0.72 | 0.39 | 7.9E-7 | 6.0E-6 | 0.4 | 0.25 |
| W118K  | 2.1E-9 | 1.5E-7 | 0.46 | 1.2 | 4.0E-7 | 1.7E-5 | 0.33 | 0.52 |
| S148K  | 6.7E-9 | 1.6E-7 | 0.32 | 0.47 | 6.7E-8 | 7.5E-6 | 0.26 | 1.1 |
| F160K  | 1.3E-8 | 5.5E-8 | 0.38 | 0.61 | 4.2E-8 | 4.8E-6 | 0.4 | 0.25 |
| I215K  | 5.7E-9 | 3.1E-7 | 0.64 | 0.34 | 1.8E-7 | 5.9E-6 | 0.22 | 0.79 |
| P191K  | 2.4E-9 | 1.5E-7 | 0.90 | 0.11 | 4.2E-8 | 4.8E-6 | 0.4 | 0.25 |
| I194K  | 5.0E-9 | 1.8E-7 | 0.86 | 0.16 | 6.9E-8 | 9.6E-6 | 0.49 | 0.02 |
| T208K  | 4.9E-9 | 1.5E-7 | 0.80 | 0.25 | 8.2E-8 | 1.2E-5 | 0.45 | 0.11 |

For Ctx GI competition results, parameters are fits of Eq. 5 to the data where $K_a$ and $K_b$ are dissociation constants and Fraction two-site is the fraction of sites corresponding to $\alpha_2\beta\epsilon\delta$ receptors, which is used to compute the ratio $\alpha_2\beta\delta_2/\alpha_2\beta\epsilon\delta$. For metocurine competition results, parameters are fits of Eq. 4 to the data where $K_a$ and $K_b$ are dissociation constants and Fraction $K_a$ is the fraction of sites with dissociation constant $K_a$. The fraction of $\alpha_2\beta\epsilon\delta$ receptors is computed as twice Fraction a, and used to compute the ratio $\alpha_2\beta\delta_2/\alpha_2\beta\epsilon\delta$. 
Table 3- ACh competition parameters for lysine mutations.

| Mutant | $K_a$  | $K_b$  | Fract. $K_a$ | $nH$ |
|--------|--------|--------|--------------|------|
| $\alpha_2\beta_2\delta$ | 6.1E-7 | NA | NA | 1.1 |
| Y111K | 5.8E-7 | NA | NA | 1.2 |
| T117K | 2.6E-7 | NA | NA | 1.1 |
| L109K | 8.3E-7 | 5.1E-5 | 0.5 | NA |
| P121K | 2.7E-6 | 1.1E-3 | 0.44 | NA |
| L50K | 2.0E-8 | 1.6E-2 | 0.44 | NA |
| T52K | 3.5E-8 | 2.3E-2 | 0.44 | NA |
| V54K | 4.8E-8 | 1.5E-2 | 0.38 | NA |
| I56K | 2.7E-8 | 1.1E-2 | 0.50 | NA |
| $\alpha_2\beta_2\delta_2$ | 3.0E-8 | 1.3E-2 | 0.55 | NA |

Parameters are fits using either the Hill equation (Eq. 1; first three rows) or two binding sites present in unequal numbers (Eq. 4; second three rows). $K_a$ and $K_b$ are dissociation constants, Fract. a is the fraction of sites with dissociation constant $K_a$ and $nH$ is the Hill coefficient. NA indicates not applicable to the fitted equation. Parameters correspond to the fitted curves in Figs. 1b and 3c.
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Figure 3
Figure 5

epsilon subunit

AChBP
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