Identification of Residues in the Adult Nicotinic Acetylcholine Receptor That Confer Selectivity for Curariform Antagonists*

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We identify residues in the ε and δ subunits of the adult nicotinic acetylcholine receptor that give the αε and αδ binding sites different affinities for the curariform antagonist dimethyl d-tubocurarine (DMT). By constructing ε-δ subunit chimeras, coexpressing them with complementary subunits, and measuring DMT binding, we identify two pairs of residues, Ile$^{58}$/His$^{66}$ and Asp$^{59}$/Ala$^{61}$, responsible for DMT site selectivity in the adult receptor. The two determinants contribute approximately equally to the binding site and interact in contributing to the site. Exchange of these residues from one subunit to the other changes the affinities of the resulting binding sites. These determinants in the adult receptor are far from those that confer site selectivity in the fetal receptor; determinants in the fetal receptor are Ile$^{116}$/Val$^{118}$, Tyr$^{117}$/Thr$^{119}$, and Ser$^{58}$/Ala$^{58}$. Thus, alternative residues confer DMT selectivity in fetal and adult acetylcholine receptors.

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The abbreviations used are: AChRs, acetylcholine receptors; DMT, dimethyl d-tubocurarine; bp, base pair.

EXPERIMENTAL PROCEDURES

Materials—Dimethyl d-tubocurarine was generously provided by Lilly. $^{3}H$-Labeled β-angutoxin was purchased from NEN Life Science Products, d-tubocurarine chloride from ICN Pharmaceuticals, Inc., and the 293 human embryonic kidney cell line from the American Type Culture Collection.

Plasmids and Mutagenesis—Mouse subunit AChR cDNAs were generously provided by Drs. Norman Davidson and John Merlie and were subcloned into the cytomegalovirus-based expression vector p7BG4 as described (1). Chimeric subunit cDNAs were constructed by bridging naturally occurring or mutagenically installed restriction sites with synthetic double-stranded oligonucleotides (1). The chimeras are designated as follows. The first letter gives the subunit from which N-terminal sequence is taken, the following number gives the position of the chimeric junction, and the final letter gives the subunit from which C-terminal sequence is taken. The chimera ε58δ36 was constructed by bridging a 35-base pair (bp) synthetic double-stranded oligonucleotide from a PfluMI restriction site in the ε subunit to a mutagenically installed SaclI site in the δ subunit. The chimeras ε43δ63ε was constructed by bridging the BstElI site in the ε subunit and the SaclI site in ε63δ with an 85-bp synthetic oligonucleotide. The chimera ε43δ65ε was constructed by bridging a 60-bp oligonucleotide from the BstElI site to the PfluMI site in the ε subunit and ligation of a 1030-bp PfluMI-PfluMI fragment prepared by digestion of the ε subunit. To construct the chimeras ε43559ε, a 3426-bp PfluMI-DraIII fragment prepared by digestion of ε43556ε was ligated with a 1900-bp AflIII-DraIII fragment from the ε subunit and a 90-bp oligonucleotide bridging the PfluMI and AflIII sites. The chimeras ε57863ε, ε58683ε, and ε59863ε were constructed from the same cassette using a 52-bp oligonucleotide that bridges the PfluMI site and a mutagenically installed Hgal I site. The point mutations ε58SH, δ59A, and ε58SHδ59A were constructed by bridging a 90-bp oligonucleotide from the PfluMI site to the ε subunit. The double mutant δ5660Aε6161D was constructed by bridging a 50-bp oligonucleotide from PfluMI to Hgal I in the ε subunit and ligating with a 300-bp Hgal I-PfluMI fragment. The double mutant γ55SHδ59A was constructed by bridging a 98-bp oligonucleotide from the PfluMI site to a mutagenically installed EagI site. The triple mutant γ117T/S161K was constructed as described (1). This triple mutant was further modified to include mutations of determinants identified in this study to γ55SHγQ58δ59A, which was constructed by bridging a 106-bp oligonucleotide from the PfluMI site to the EcoRV site. All constructs were confirmed by dideoxy sequencing.

Expression of Mutant Receptors and Ligand Binding Measurements—Human embryonic kidney cells were transfected with mutant or wild-type AChR subunit cDNAs using calcium phosphate precipitation as described (1). Three days after transfection, intact human embryonic kidney cells were harvested by gentle agitation in phosphate-buffered saline plus 5 mM EDTA. DMT binding to intact cells was measured by competition against the initial rate of $^{3}H$-β-angutoxin binding (11). After harvesting, the cells were briefly centrifuged, resuspended in potassium Ringer high solution, and divided into aliquots for DMT binding measurements. Potassium Ringer solution contains 140 mM KCl, 5.4 mM NaCl, 1.8 mM CaCl$_2$, 1.7 mM MgCl$_2$, 25 mM HEPES, and 30 mg/liter bovine serum albumin adjusted to pH 7.4 with 10–11 mM NaOH. Specified concentrations of DMT were added 30 min prior to the addition of $^{3}H$-β-angutoxin, which was allowed to bind for 90 min to occupy approximately half of the surface receptors. Binding was terminated by the addition of 2 ml of potassium Ringer solution containing 300 μM d-tubocurarine chloride. Cells were then harvested by filtration through Whatman GF/B filters using a Brandel Cell Harvester and washed four times with 3 ml of potassium Ringer solution.
Prior to use, filters were soaked in potassium Ringer solution containing 4% skim milk for a minimum of 2 h. Nonspecific binding was determined in the presence of 10 mM carbachol. The total number of α-bungarotoxin sites was determined by incubation with toxin for 120 min. The initial rate of α-bungarotoxin binding was calculated as described (11) to yield fractional occupancy by DMT. Binding measurements were analyzed according to either the monophasic Hill equation (Equation 1) or the sum of two distinct binding sites (Equation 2),

\[
1 - Y = 1/(1 + [\text{ACh}]/K_{\text{app}}) \quad (\text{Eq. 1})
\]

\[
1 - Y = \text{frac}_1/(1 + [\text{ACh}]/K_1) + \text{frac}_2/(1 + [\text{ACh}]/K_2) \quad (\text{Eq. 2})
\]

where \(Y\) is fractional occupancy by DMT, \(n\) is the Hill coefficient, \(K_{\text{app}}\) is an apparent dissociation constant for a monophasic binding profile, \(K_1\) and \(K_2\) are intrinsic dissociation constants for two binding sites, \(\text{frac}_1\) is the fraction of sites with dissociation constant \(K_1\), and \(\text{ACh}\) is acetylcholine. For binding determinations from single experiments, fitted parameters and standard errors were obtained using the program UltraFit (BIOSOFT). For multiple experiments, means ± S.D. of the individual fitted parameters are presented (Table 1).

RESULTS

\(\varepsilon-d\) Subunit Chimeras—Previous work showed that the pair of equivalent residues Tyr\(^{117}\)/Thr\(^{119}\) are major determinants of DMT selectivity in the fetal AChR (1). Tyr\(^{117}\) contributes to high affinity of the \(\varepsilon\) subunit site, whereas Thr\(^{119}\) contributes to low affinity of the \(\alpha\) subunit site. Because serine occupies the equivalent position in the \(\varepsilon\) subunit and high affinity requires an aromatic side chain, the \(\alpha\) subunit site is not expected to bind DMT with high affinity. To determine the origin of high affinity conferred by the \(\varepsilon\) subunit, we constructed a series of \(\varepsilon-d\) subunit chimeras (Fig. 1, A and B), coexpressed them with complementary subunits, and measured DMT binding by competition against the initial rate of \(\alpha-\)\(^{125}\)I-bungarotoxin binding.

Selectivity of the adult AChR for DMT is illustrated in Fig. 1C; distinct affinities of the \(\alpha\) and \(\varepsilon\) sites are clearly resolved, with the two-site fit disclosing dissociation constants differing by 70-fold (Table I). Substituting \(\varepsilon\) sequence into the N-terminal 63 positions of the \(\delta\) subunit increases DMT affinity to a position that conferred by the pure \(\varepsilon\) subunit (Fig. 1C, \(\varepsilon63\)). Conversely, substituting \(\delta\) sequence between positions 43 and 63 of the \(\varepsilon\) subunit decreases affinity to approach that of the pure \(\delta\) subunit (Fig. 1C, \(\delta63\)). Thus, determinants of DMT selectivity in the adult AChR are located in the major extracellular domain between residues 43 and 63 of the \(\varepsilon\) subunit and equivalent residues of the \(\delta\) subunit.

Expanding the Window of Selectivity with 8L121K—Because we anticipated multiple contributions to DMT selectivity, we sought to increase the window of selectivity to help discern small affinity changes. Recent studies in our laboratory revealed that the mutation 8L121K decreases DMT affinity for the \(\alpha\) site while retaining good levels of expression (19). When incorporated into the adult AChR, 8L121K markedly expands the selectivity window from 70- to ∼2000-fold (Fig. 2B). The broad plateau allows determination of the fraction of each site and clear resolution of the dissociation constants for the two sites. The apparent fraction of sites with high affinity is ~0.6 in the AChR containing 8L121K. Because the receptors lacking the \(\delta\) subunit (i.e. \(\alpha_3\beta_2\)) used in this study do not express significantly at 37 °C, the binding profile should arise entirely from \(\alpha_3\beta_2\)8L121K receptors, and the fraction of each site should equal 0.5. Thus, the apparent fractional of 0.6 suggests that the intrinsic rate of toxin binding is somewhat slower at the 8L121K site compared with the native \(\alpha\) or \(\varepsilon\) sites.

We further localized selectivity determinants by constructing \(\varepsilon-d\) subunit chimeras that maintained one junction at position 43, but shifted the other junction from position 63 toward the N terminus (Fig. 2A). When coexpressed with \(\alpha\), \(\beta\), and 8L121K subunits, the chimera ε43595e confers low, \(\delta\)-like affinity (Fig. 2B), as observed for ε43563e (Fig. 1C). The resulting binding profile is the sum of contributions of the site formed by \(\alpha\) and 8L121K and that formed by \(\alpha\) and ε43595e. We determined the dissociation constant of the site formed by \(\alpha\) and ε43565e by fitting a two-site equation (Equation 2) to the data; the fit reveals a dissociation constant of 16 μM for the site formed by \(\alpha\) and ε43559e, indistinguishable from that of the pure \(\alpha\) site (Table I). The dissociation constant of 0.39 μM for the α8L121K site agrees with that obtained for the same site in receptors containing the wild-type \(\varepsilon\) subunit (Table I). These results narrow the region containing selectivity determinants to between residues 43 and 60 of the \(\varepsilon\) subunit.

Moving the chimera junction three more positions toward the N terminus, yielding ε43566e (Fig. 2A), increases DMT affinity to that conferred by the pure \(\varepsilon\) subunit (Fig. 2B). Observation of pure \(\varepsilon\)-like affinity suggests that the region from residues 44 to 56 contains no additional selectivity determinants. These results narrow the region containing selectivity determinants to between residues 56 and 60 of the \(\varepsilon\) subunit; the corresponding \(\varepsilon\) sequence is GHD, whereas the equivalent \(\delta\) sequence is DHA.

To determine which of the three remaining pairs of residues confer DMT selectivity, we constructed ε57663e, ε58663e, and ε59663e (Fig. 3A). The chimera ε57663e confers the same low \(\delta\)-like affinity observed with ε43563e, indicating that the Gly...
TABLE I
DMT binding parameters for receptors containing wild-type or mutant ε subunits

| Mutant                  | $K_a$ (μM) | $K_D$ (μM) | $\text{frac}_{C_a}$ | $K_{app}$ (μM) | $n_H$ |
|-------------------------|------------|------------|----------------------|---------------|-------|
| Coexpressed with 0L121K|            |            |                      |               |       |
| Wild-type ε             | 0.11 ± 0.01 (3) | 432 ± 72.3 | 0.59 ± 0.01 | 3.04 ± 1.1 | 0.28 ± 0.04 |
| ε4356ε                  | 0.28 ± 0.04 (1) | 372 ± 92.0 | 0.66 ± 0.01 | 2.42 ± 0.7 | 0.38 ± 0.06 |
| ε4359ε                  | 16.4 ± 2.1 (1) | 389 ± 68.0 | 0.62 ± 0.02 | 43.0 ± 5.2 | 0.72 ± 0.06 |
| ε5766ε                  | 21.7 ± 3.7 (2) | 493 ± 89.9 | 0.51 ± 0.02 | 105.0 ± 15.3 | 0.68 ± 0.07 |
| ε5866ε                  | 1.2 ± 0.31 (1) | 369 ± 63.2 | 0.43 ± 0.03 | 34.7 ± 11.4 | 0.41 ± 0.05 |
| ε5966ε                  | 0.16 ± 0.02 (1) | 632 ± 190.1 | 0.64 ± 0.03 |               |       |
| ε56H                    | 1.4 ± 0.1 (1) | 352 ± 58.6 | 0.64 ± 0.03 | 8.47 ± 1.9 | 0.46 ± 0.05 |
| ε5D9A                   | 1.1 ± 0.2 (1) | 360 ± 115.6 | 0.61 ± 0.02 | 11.37 ± 1.9 | 0.38 ± 0.03 |
| ε56H/e55A               | 18.1 ± 2.6 (1) | 286 ± 37.1 | 0.51 ± 0.02 | 75.52 ± 6.2 | 0.75 ± 0.05 |

| Mutant                  | $K_a$ (μM) | $K_D$ (μM) | $\text{frac}_{C_a}$ | $K_{app}$ (μM) | $n_H$ |
|-------------------------|------------|------------|----------------------|---------------|-------|
| Wild-type δ             | 0.17 ± 0.06 (10) | 12.5 ± 0.04 | 0.50 | 1.56 ± 0.41 | 0.54 ± 0.03 |
| ε63δ                    | 0.11 ± 0.06 (4) | 0.64 ± 0.27 | 0.50 | 0.28 ± 0.07 | 0.90 ± 0.09 |
| ε4366ε                  | 0.74 ± 0.11 (1) | 9.38 ± 1.3 | 0.50 | 2.44 ± 0.16 | 0.75 ± 0.01 |
| ε5766ε                  | 6.42 ± 1.6 (2) | 20.5 ± 5.06 | 0.50 | 11.50 ± 0.69 | 0.92 ± 0.05 |
| ε5866ε                  | 1.63 ± 0.37 (1) | 8.40 ± 1.9 | 0.50 | 3.70 ± 0.29 | 0.90 ± 0.06 |
| ε5966ε                  | 0.61 ± 0.07 (1) | 8.56 ± 1.0 | 0.50 | 2.30 ± 0.13 | 0.76 ± 0.03 |
| ε56H                    | 2.87 ± 0.27 (1) | 11.69 ± 1.09 | 0.50 | 5.79 ± 0.16 | 0.91 ± 0.03 |
| ε5D9A                   | 4.27 ± 0.85 (1) | 12.2 ± 2.40 | 0.50 | 7.29 ± 0.34 | 0.95 ± 0.04 |
| ε58H/e55A               | 7.91 ± 1.03 (1) | 27.9 ± 3.6 | 0.50 | 14.90 ± 0.47 | 0.92 ± 0.02 |

vectors Asp 60 and Asp 63 elicit a partial shift toward high affinity, and ε56H/e55A elicits a complete shift to pure ε-like affinity (Fig. 3B). These results suggest that two pairs of adjacent residues confer DMT selectivity in the adult receptor, Leu65/His66 and Asp65/Ala66.

Point Mutations of Selectivity Determinants—To determine whether the selectivity determinants identified using chimeras
are solely responsible for DMT selectivity in the adult receptor, we constructed point mutations in the ε subunit, coexpressed them with α, β, and δL121K subunits, and measured DMT binding. As observed with the chimera ε57863ε, the single point mutations ε585H and D595A partially decrease the affinity of the αε site to approach that of the δε site (Fig. 4A). Moreover, the double mutation ε1558H/D595A fully decreases the affinity to that conferred by both the chimera ε57863ε and the pure δε subunit (Fig. 4A). We determined the dissociation constant of the site formed by α and ε1558H/D595A by fitting a two-site equation (Equation 2) to the data; the fit revealed a dissociation constant of 20 μM for the site formed by α and ε1558H/D595A, close to that of the native εδ site (Table I). Thus, the pair of residues Ile658 and Asp563 fully account for DMT site selectivity in the adult receptor.

Having defined selectivity determinants using δL121K to increase the window of selectivity, we sought to confirm the contributions of Ile658 and Asp563 in the presence of complementary wild-type subunits. Again, the ε single mutations partially decrease affinity, and the double mutation fully decreases affinity to that of the pure δ subunit (Fig. 4B). Fitting the Hill equation to the data for the double mutant reveals a single class of sites with a dissociation constant of 14 μM (Table I).

Exchange of Selectivity Determinants between the ε and δ Subunits—To further confirm that the pairs of equivalent residues Ile658/His660 and Asp563/Ala567 are solely responsible for DMT selectivity in the adult receptor, we expressed the double mutants ε1558H/D595A and δH601/δ661D, alone or together, and measured DMT binding. Receptors containing δH601/δ661D bind DMT with a single high affinity dissociation constant, approaching that conferred by the ε subunit (Fig. 4C and Table I). As just described, receptors containing ε1558H/D595A bind DMT with a single low affinity dissociation constant, approaching that conferred by the δ subunit. Moreover, incorporating both the ε and δ double mutants into a single receptor mimics the selective binding of DMT to the wild-type adult receptor (Fig. 4C). Thus, the pair of equivalent residues Ile658/His660 and Asp563/Ala567 account entirely for DMT selectivity in the adult receptor.

Point Mutations of Ser117—The experiments using ε-δ subunit chimeras were motivated by the presence of serine at position 117 of the ε subunit and the observation that tyrosine at the equivalent position of the γ subunit is associated with high affinity for curariform antagonists (1, 9). To determine whether Ser117 is nevertheless close enough to affect DMT binding, we mutated Ser117 to threonine, valine, tyrosine, phenylalanine, tryptophan, and arginine. The phenylalanine mutation slightly enhances the affinity of DMT for the αε site, whereas the threonine and tyrosine mutations are without effect (Table II). The remaining aromatic mutation, tryptophan, slightly decreases affinity to about the same extent observed with the valine mutation. The arginine mutation shows the greatest decrease in affinity. Thus, four of the six mutations at Ser117 produce small changes in the affinity of the αε site, with little effect on the affinity of the δε site. The side chain specificity of position 117 of the ε subunit, however, differs markedly from that observed with point mutations of Tyr117, where, for example, γY117W enhances affinity 30-fold compared with γY117S (9). The relatively small effects of these
mutations on DMT binding to the α site suggests that Ser**117** is close to the site of binding, but it does not contribute to high affinity for DMT.

**Point Mutations in the γ Subunit, Met**58** and Gln**59**.—Because the determinants identified in this study differ from those that confer high affinity in the homologous γ subunit, we mutated the equivalent residues in the γ subunit, Met**58** and Gln**59**, to their counterparts in the low affinity δ subunit. The site formed by α and γ M58I/Q59A maintains high affinity for DMT, with the dissociation constant of 0.23 μM close to that of the native αγ site (Table III). Thus, the determinants identified in this study do not affect the contribution of the γ subunit to DMT binding.

We considered the possibility that γ M58I/Q59A does not decrease DMT affinity because the previously identified determinants of high affinity (Ile**516**, Tyr**717**, and Ser**161**) are still present. Thus, we constructed the resulting double mutant (γ M58I/Q59A) with α, β, and δ subunits and measured DMT binding. The site formed by α and γ M58I/Q59A maintains high affinity for DMT, with the dissociation constant of 0.23 μM close to that of the native αγ site (Table III). Thus, the determinants identified in this study do not affect the contribution of the γ subunit to DMT binding.

**DISCUSSION**

These experiments identify residues in the ε and δ subunits that give the two binding sites of the adult mouse AChR different affinities for the curariform antagonist DMT. Previous work identified a different set of residues in the γ and δ subunits that confer site selectivity in the fetal mouse AChR (1). Two sets of determinants were identified, with each set flanking the disulfide loop common to all members of the AChR superfamily; the pre-disulfide set is Ile**516**, Val**511**, and Tyr**717**; Thr**519**, and the post-disulfide set is Ser**161**, Lys**163**. By contrast, selectivity determinants in the adult receptor are far from these in the linear sequence and comprise Ile**526**, His**560**, and Asp**569**, Ala**641**; showing that alternative residues confer DMT selectivity in fetal and adult receptors. The results support a basic scaffold hypothesis because selectivity can be exchanged between the ε and δ subunits by exchanging a small number of residues at equivalent positions of the primary sequence.

The three pairs of selectivity determinants identified in the fetal receptor, the pair Tyr**717**, Thr**519** makes the greatest contribution to DMT selectivity (1). Studies of side chain specificity indicate that Tyr**717** stabilizes one of two quaternary nitrogens in DMT through a π-cation interaction (9). Because serine is present at position 117 of the high affinity ε subunit, we reasoned that the source of high affinity conferred by the ε subunit should be elsewhere. Our results of point mutations of Ser**117** reveal relatively small changes in the affinity of the αε site, indicating that although Ser**117** is not the origin of high affinity for DMT, it may nevertheless be close to the site of binding. In contrast to the π-cation stabilization observed in the fetal receptor, DMT selectivity appears to owe to electrostatic forces in the adult receptor. High affinity of the αε site results from an isoleucine-aspartic acid pair, whereas low affinity of the αδ site results from a histidine-alanine pair.

**Difference of Two Mutations of the determinants, ε58H or ε59A, partially decreases affinity, whereas mutation of both determinants fully decreases affinity to that conferred by the native δ subunit. The sum of the contributions of the single mutations slightly exceeds that of the double mutation (Fig. 4B), pointing to some sort of interaction between these determinants. High affinity of the αε site may owe to electrostatic forces between Asp**64** and one of the two quaternary nitrogens in DMT. If the local pH renders His**61** positively charged, low affinity of the αδ site may owe to electrostatic repulsion of a positive charge in DMT. Preliminary measurements with the mutation ε58K reveal decreased affinity similar to that of ε58H. Alternatively, other nearby residues, perhaps with aromatic side chains, may directly stabilize DMT, and the determinants that we have identified may affect their interaction with DMT.

The selectivity determinants in the adult receptor belong to **Epsilon**

| Mutant | $K_A$ | $K_B$ | $K_{app}$ | $n_H$ |
|--------|-------|-------|-----------|-------|
| Wild-type (αβγδ) | 0.134 ± 0.016 | 6.89 ± 0.81 | 12.88 ± 1.95 | 9.13 ± 0.59 | 0.99 |
| γM58I/Q59A | 0.225 ± 0.034 | 9.13 ± 0.59 | 0.99 |
| γM58I/Q59D/γY117T/γY117T/γS161K | 6.12 ± 0.38 | 0.92 |

**FIG. 5.** Sequence alignments of ε, δ, and γ subunits in the region containing DMT selectivity determinants. Selectivity determinants are highlighted by enlarged letters.
one of the four loops identified to contribute to the non-α subunit portion of the ligand-binding interface (12, 20). Local sequences of this loop are compared across species for the ε, δ, and γ subunits (Fig. 5). The two determinants are highly conserved among ε and γ subunits, with the first determinant an invariant isoleucine among ε subunits and methionine in all γ subunits except Torpedo. The second determinant is an acidic group in ε subunits and a neutral glutamine in γ subunits. By contrast, the determinants are not as well conserved among δ subunits, with the proton acceptor histidine or polar glutamine at the first position and small but variable residues at the second.

The selectivity determinants are near other residues known to contribute to the ligand-binding site. The invariant pair Trp55/Trp557 was first identified by photoaffinity labeling with 3H-labeled d-tubocurarine (13) and has been shown to contribute to ligand affinity by mutagenesis (14, 15). Two residues carboxyl-terminal to Trp55/Trp557 is the pair Glu57/Asp59, which contributes to agonist selectivity in a state-specific manner, preferentially affecting the desensitized state (12). Equivalent to this pair in neuronal subunits is Thrβ5α/Lysβ5α, which contributes to higher affinity of dihydro-β-erythroidine and neuronal bungarotoxin for αβγε and αβγδ sites, respectively.

Previous studies showed that tyrosines in the juxtaposed α and γ subunits, Tyrε198 and Tyrγ1717, stabilize DMT through symmetrical π-cation interactions (9). By studying a series of mutations in both subunits, the contributions of each residue were found to be approximately equal and additive, indicating that DMT bridges the αγ subunit interface. DMT may also bridge the αε subunit interface in the receptor, but with a different point of attachment in the ε subunit. The determinants we identify may also affect attachment of DMT to the α subunit portion of the binding site.

The high degree of homology among AChR subunits suggests that the polypeptide chains of each subunit fold into similar basic scaffolds. Support for this basic scaffold hypothesis comes from the observation that site selectivity of agonists and antagonists can be exchanged between the γ and δ subunits by exchanging a small number of residues at equivalent positions of the primary sequence (1, 12, 17). Nearly interchangeable alterations in affinity are observed for the two pairs of selectivity determinants in the ε and δ subunits identified in this study; this would not be expected for a rigid ligand such as DMT unless the determinants are in the same positions at both the αε and αδ subunit interfaces. Thus, the identified determinants probably occupy equivalent positions within the ε and δ scaffolds, supporting the basic scaffold hypothesis.

In addition to showing that different residues confer high affinity to the ε and γ subunits, our experiments reveal an additional difference between these subunits. In particular, neither γM58I/γQ59D nor γM58I/γQ59A affects the contribution of the γ subunit to the DMT-binding site, whereas the corresponding mutations in the ε subunit elicit profound changes in DMT affinity. Thus, while the polypeptide scaffolds in the ε and γ subunits are likely to be similar, particularly since they are the most homologous pair of AChR subunits, a structure unique to the γ subunit prevents residues placed at positions 58 and 59 of the γ subunit from contributing to DMT binding.

One can reasonably ask how two completely different sets of residues confer selectivity for the same ligand. Considering first the low affinity αδ site, DMT binds with a dissociation constant of 10 μM, which of course requires some stabilizing interactions. Perhaps these include stabilization of one of the two quaternary nitrogen atoms in DMT by Tyr198 in the juxtaposed α subunit (14, 18) plus stabilization of the hydrophobic and hydrophilic faces of DMT by as yet unidentified residues within the αδ subunit interface. Considering the high affinity αε site, DMT is still stabilized by structures common to the αδ and αγ sites, but its second quaternary nitrogen is within reach of Tyr1717, which provides additional stabilization and high affinity. The high affinity αε site, on the other hand, contains serine at position 117, which is within reach of the second quaternary nitrogen of DMT, but provides no further stabilization. Instead, DMT appears to position its second quaternary nitrogen close to Ile558 and Asp590 to bind with high affinity and a dissociation constant of ~0.1 μM. Thus, at the various αγ, αδ, and αε binding sites, DMT chooses among possible sources of stabilization within the binding cleft and associates with the most favorable stabilizing residues.

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