Contributions of Torpedo Nicotinic Acetylcholine Receptor γTrp-55 and δTrp-57 to Agonist and Competitive Antagonist Function*

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Results of affinity-labeling studies and mutational analyses provide evidence that the agonist binding sites of the nicotinic acetylcholine receptor (nAChR) are located at the α-γ and α-δ subunit interfaces. For Torpedo nAChR, photoaffinity-labeling studies with the competitive antagonist d-[3H]tubocurarine (dTC) identified two tryptophans, γTrp-55 and δTrp-57, as the primary sites of photolabeling in the non-α subunits. To characterize the importance of γTrp-55 and δTrp-57 to the interactions of agonists and antagonists, Torpedo nAChRs were expressed in Xenopus oocytes, and equilibrium binding assays and electrophysiological recordings were used to examine the functional consequences when either or both tryptophans were mutated to leucine. Neither substitution altered the equilibrium binding of dTC. However, the δW57L and γW55L mutations decreased acetylcholine (ACh) binding affinity by 20- and 7,000-fold respectively. For the wild-type, γTrp-55 and δTrp-57, as the primary sites of photolabeling in the non-α subunits. For the wild-type, the equilibrium binding of dTC was characterized by Hill coefficients of 1.8, 1.1, and 1.7. For the γW55L mutant, dTC binding at the α-γ site acts not as a competitive antagonist but as a coactivator or partial agonist. These results establish that interactions with γTrp-55 of the Torpedo nAChR play a crucial role in agonist binding and in the agonist-induced conformational changes that lead to channel opening.

The nicotinic acetylcholine receptor (nAChR)1 from Torpedo electric organ and vertebrate skeletal muscle is a pentameric transmembrane protein composed of four homologous subunits with a stoichiometry of α₂βγδ (reviewed in Refs. 1–3). The nAChR contains two binding sites for agonists and competitive antagonists, located at the α-γ and α-δ subunit interfaces (4, 5). The two sites are nonequivalent, and many competitive antagonists bind with high affinity to only one of the sites (6, 7). Affinity labeling and mutational analyses provide evidence that amino acids from three discrete regions of α subunit primary structure and from three (or more) regions of the γ (or δ) subunit contribute to the structure of the binding sites (reviewed in Refs. 8 and 9).

Several experimental approaches have identified residues in both γ and δ subunits that contribute to the binding sites for agonists or competitive antagonists. Photoaffinity labeling using d-[3H]tubocurarine (dTC), a competitive antagonist, and [3H]nicotinic, an agonist, established that γTrp-55 is located near the agonist binding site in Torpedo nAChR (10, 11), and [3H]dTC also reacted with δTrp-57, the corresponding position in the δ subunit, as well as with γTyr-111/γTyr-117 (12). Substitution of γTrp-55 by leucine (γW55L) caused a 10-fold decrease in dTC potency as an inhibitor of ACh-induced currents for Torpedo nAChRs expressed in Xenopus oocytes (13). A heterobifunctional cross-linker —9 Å in length cross-linked αCys-192/αCys-193 in Torpedo nAChR to a residue in the δ subunit identified as δAsp-180 (14, 15), and the mutation δD180N in the δ subunit of mouse nAChR caused substantial decreases in agonist, but not antagonist, binding affinities (16–18). Analysis of binding properties of embryonic mouse nAChRs containing chimeras between γ and δ subunits led to the identification of three positions in the γ subunit (Ile-116, Tyr-117, and Ser-161) and the corresponding residues in the δ subunit (Ser-118, Thr-119, and Lys-163), which can account for the binding selectivity of the competitive antagonist dimethyl-d-tubocurarine (metocurine (19)), whereas for the agonist carbamylcholine, the primary determinants of site selectivity were γLys-34/δSer-36 and γPhe-172/δIle-178 (20). For the adult mouse nAChR (α₂βγδ), a similar analysis of chimeric ε-δ subunits identified amino acids in another region of subunit primary structure (ε-δIle-58/δHis-60 and εAsp-59/δ Ala-61) as determinants of metocurine site selectivity (21).

In this report we examine the contributions of γTrp-55 and δTrp-57 as determinants of agonist binding and channel gating as well as determinants of competitive antagonist function. γTrp-55 and δTrp-57 were mutated to leucine, and the interaction of agonists and antagonists were examined using both binding assays and electrophysiological recording. Concentration-dependent inhibition of 125I-α-bungarotoxin (α-BgTx) binding by agonists and antagonists was studied using wild-type and mutant Torpedo nAChRs in membranes isolated from Xenopus oocyte homogenates. In parallel experiments, we studied the activation of wild-type and mutant nAChRs by ACh using a two-electrode voltage clamp. Our results establish that the mutation γW55L has no effect on dTC equilibrium binding affinity. However, the mutation has a profound effect on ACh binding and on the gating of the ion channel. Furthermore, for the γW55L mutant nAChR, dTC acts not as an antagonist when bound to its high affinity site but as a coactivator with ACh.

**EXPERIMENTAL PROCEDURES**

*MATERIALS—ACh, dTC, tetramethylammonium, phenyltrimethylammonium, suberylcholine, nicotine, and gallamine were obtained from Sigma. Epibatidine and dihydro-β-erythroidine were obtained from Sigma-RBI (Natick, MA), and pancuronium was from Organon Inc. (W. Orange, NJ). α-BgTx was from Biotoxins Inc. (St. Cloud, FL). 125I-α-

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Bgtx (400–600 Ci/mmol) was prepared using iodine (Pierce) and Na[125I] as described (22). Na[125I] and [35S]methionine/cysteine were from PerkinElmer Life Sciences. Affinity-purified rabbit antibodies against α-BgTx were kindly provided by Dr. Robert Sealloc (University of North Carolina, and motocure and 13-iodo-dTC were donated by Dr. Peter Cohen (23).  

In Vitro Transcription and Expression in Xenopus Oocytes—SP64-based plasmids (pMXT) with cDNAs encoding wild-type α, γ, δ, and the γ55L subunits were gifts from Dr. Michael M. White, and the cDNA (in plasmid SP64) encoding the wild-type β subunit was donated by Dr. Henry Lester. Sequence analysis of the δ55L mutant cDNA (from Dr. White) revealed a point deletion 353 bases 3′ to the δ55L mutation was subsequently introduced into pMXT and resulted in a truncated form of the subunit. Therefore, the full-length δ55L mutant subunit was prepared by subcloning a NheI fragment (present in the vector) and BamHI fragment (containing the desired mutation (δ55L) but excluded the point deletion into the wild-type δ subunit cDNA from which the corresponding region had been excised. cDNAs were linearized with either XbaI (for wild-type α, β, γ, δ, γ55L, and δ55L mutant subunits) or FspI (for the wild-type β subunit). In vitro transcription reactions were carried out in transcription buffer (Promega) containing 40 mM Tris (pH 7.5), 6 mM MgCl2, 2 mM spermidine, and 10 mM NaCl. Linear cDNAs (5–10 μg) were incubated with 10 μM dithiothreitol, NTPs (1 mM each except GTP, which was 0.2 mM), 0.6 mM diguanosine triphosphate (Amersham Pharmacia Biotech), 100 units of RNasin (Promega), and 0.1 units of aprotinin/ml (pH 7.6). Membranes were isolated as described (22) by differential and sucrose density centrifugation. Membranes were then used to prepare membranes containing either surface or total receptors were then suspended in HB buffer supplemented with 1% Triton X-100. After solubilization, the extracts were treated with 1% Immunoprecip (Life Technologies, Inc.) at 4 °C for 20 min and centrifuged for 1 min in an Eppendorf microcentrifuge. Immunoprecipitin-treated supernatants were then collected and incubated with an excess amount of rabbit anti-α-BgTx antibody at 4 °C overnight. After overnight incubation, immunoprecipitin (1%) was added to the nAChR-antibody complex and incubated at 4 °C for 30 min. The samples were then spun in a microcentrifuge for 1 min, and the supernatants were discarded. The immunoprecipitate was washed four times with HB buffer containing 1% Triton X-100, 0.1% SDS, and 0.5% bovine serum albumin and once with HB buffer containing 0.1% SDS and 0.05% Triton X-100 (without bovine serum albumin) and once with HB buffer containing 0.1% SDS followed by incubation in 50 μl of SDS-polycrylamide gel electrophoresis sample buffer and incubated at room temperature for 20–30 min. The samples were then electroblotted on an 8% SDS-polyacrylamide gel. After staining with Coomassie Blue and destaining, the gels were soaked in Amplify (Amersham Pharmacia Biotech) for 30 min, dried at 65 °C for 2 h, and exposed to x-ray film at –80 °C for 24 h.  

Sucrose Density Gradient Analysis of nAChRs in Oocytes—To characterize surface nAChRs, 15–30 injected intact oocytes were incubated with 2.5 nM 125I-α-BgTx for 2 h followed by a wash, and oocyte membranes were then prepared as described above. The membranes were then solubilized with 1% Triton X-100 in a final volume of 200 μl of HB buffer and centrifuged in a Ti 42.2 rotor at 35,000 rpm for 20 min. The supernatants (~180 μl) were used for sedimentation analysis. For total nAChRs, oocyte membranes containing surface nAChRs prelabeled by 125I-α-BgTx were then incubated with 2.5 μM 125I-α-BgTx for another 2 h. After a 2-h incubation, membranes were washed and solubilized as described above. 180-μl samples from 10–15 oocytes containing either surface or total receptors were sedimented on a 10-ml 3–30% sucrose gradient (150 mM NaCl, 5 mM EDTA, 50 mM Tris pH 7.6, 1% Triton X-100, and 1 m M dithiothreitol) in a SW 40 Ti rotor (Beckman) for 2 h at 4 °C. After 10 min, 125I-α-BgTx-labeled surface receptors in 200-μl extracts from Torpedo membranes prelabeled with 125I-α-BgTx were used as the control for monomeric (9.5 S) and dimeric nAChR (13 S), and free 125I-α-BgTx (1.7 S), alkaline phosphatase (6.1 S), and β-galactosidase (15.9 S) were used as markers for sedimentation coefficients. Fractions (200 μl) were collected and counted on a γ counter.  

Electrophysiology—Currents elicited by ACh were measured using a standard two-electrode voltage clamp (Oocyte Clamp QC-725B, Warner Instrument Corp.) at a holding potential of ~70 mV. Electrodes were filled with 3 M KCl and had resistances of 0.5–1.5 megohms. The recording chamber (about 150 μl in volume) was perfused continually by gravity with low Ca2+ ND96 (plus 1 μM atropine, pH 7.6). Appropriately, concentrations of ACh (or other agonists) in the absence or presence of antagonists were applied through solenoid valves into the recording chamber for 3–5 s. For some experiments, oocytes were perfused with dTC by perfusing the oocytes for 1–2 min with dTC in low Ca2+ ND96 before application for 5 s of solution containing ACh with the same concentration of dTC.  

Data Analysis—The concentration-dependent inhibition of 125I-α-BgTx binding by agonists and antagonists was fit according to two models as follows:  

\[ f = 100(1 + [(\text{IC}_{50}^\text{a})/X]) \]  

(Eq. 1)  

\[ f = (50/[1 + ((X/K_P) + (50/[1 + (X/K_{IC}]))]) \]  

(Eq. 2)  

where [X] is the concentration of inhibitor, n is the Hill coefficient, and IC_{50}^a is the inhibitor concentration reducing the initial rate of 125I-α-BgTx binding by 50% and [X] is the concentration of competing ligand, K_P and K_{IC} are the ligand affinities for the high and low affinity binding sites, respectively. This equation is based on the assumption that α-BgTx binds at equal rates to the two sites.
The dose-dependent inhibition of ACh-induced currents by antago-

-ists was fitted according to Equation 1 above. SigmaPlot (Jandel Scien-
tific) was used for nonlinear least squares fit of the data, and the S.E. of the parameter fits are indicated in the Tables.

**RESULTS**

**Influence of γW55L and δW57L on the Binding of d-Tubocurar-
arine and Acetylcholine—Torpedo nAChRs were expressed in

*Xenopus* oocytes by injection of cRNAs encoding α, β, γ, and δ subunits. We characterized the equilibrium binding of antago-

-nists and agonists by their inhibition of 125I-α-BgTx binding to

*Torpedo* nAChRs in membranes isolated from oocyte homoge-

-nates for wild-type nAChRs (W55L (Ref. 13

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), δW57L (Ref. 13

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), or both mutant sub-

-units (αβγδ

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) for native Torpedo membranes (○). Assay aliquots (100 μl) of Torpedo membranes con-

-tained 50 fmol of nAChR and membranes from 3 un.injected oocytes and were treated with 100 mM disopropylphosphofluoridate to inactivate cholinesterase. Insets, dTC (A) and ACh (B) binding to γ-less (●), αβδ

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), and δ-less (○, αβγδ

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) nAChRs compared with wild-type (○). For each data set, the data points represent the mean ± S.D. of triplicate samples from a single experiment representative of 2–4 experiments. Solid curves are calculated from the parameters of Table I for the two-site model (Experimental Procedures,” Equation 2).

For receptor activation, concentration-response curves for ACh and other agonists were fit to the following equation.

\[
III_{\text{max}} = \frac{1 + (K_E/[ACh])^n}{1 + (K_E/[ACh])^n} \quad \text{(Eq. 3)}
\]

where I and \(I_{\text{max}}\) are the currents at a given concentration of ACh and the maximal value, respectively, and \(K_E\) is the concentration of ACh required for half-maximal current. Because high concentrations of ACh do not result in a concentration-independent maximal response (due to desensitization and/or channel block), the Hill coefficients (\(n_H\)) for the agonist dose-response relations were estimated from the slope of plots of log I versus log[agonist] at currents less than 20% of the maximal response for each agonist.

The dose-dependent inhibition of ACh-induced currents by antago-

-nists was fit according to Equation 1 above. SigmaPlot (Jandel Scien-
tific) was used for nonlinear least squares fit of the data, and the S.E. of the parameter fits are indicated in the Tables.

The effects of the γW55L and δW57L mutations on ACh binding were clearly different than that observed for nAChRs lacking either γ subunit (γ-less, αβδ

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, IC\(_{50}\) = 0.8 μM) or δ subunit (δ-less, αβγ

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, IC\(_{50}\) = 0.3 μM), which were characterized by inhibition curves similar to wild-type (Fig. 1B, inset and Table I). As judged by inhibition of 125I-α-BgTx binding, ACh binding to *Torpedo* nAChRs expressed in oocytes also differed from the binding to native nAChRs in *Torpedo* membranes (IC\(_{50}\) = 25 nM and a Hill coefficient of 0.9 (Fig. 1B)).

The observed equilibrium binding reflects the binding affinity of the ACh sites in the desensitized nAChR and the confor-
mational equilibrium between resting and desensitized states. To test whether the leucine substitution had a predominant effect on the latter parameter, we examined the effect of proa-
difen, a desensitizing noncompetitive antagonist (24), on the ACh equilibrium binding function. For wild-type and αβγδ

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,
nAChR Agonist Binding Determinant

Table 1
Equilibrium binding parameters for dTC and ACh

|                | Iceq | n  | Ks | Kl |
|----------------|------|----|----|----|
|                |      |    |    |    |
| dTC            |      |    |    |    |
| α₂βγδ          | 4.4 ± 0.3 × 10⁻⁷ | 0.52 ± 0.01 | 5.0 ± 1.0 × 10⁻⁶ | 3.9 ± 1.0 × 10⁻⁶ |
| α₂βγδ₂         | 7.9 ± 0.7 × 10⁻⁷ | 0.90 ± 0.06 | 2.7 ± 0.7 × 10⁻⁸ | 7.9 ± 1.6 × 10⁻⁷ |
| α₂βγδ₂₂        | 1.4 ± 1.0 × 10⁻⁷ | 0.65 ± 0.03 | 3.9 ± 0.4 × 10⁻⁸ | 4.8 ± 0.5 × 10⁻⁸ |
| α₂βγδ₂₆       | 3.7 ± 0.8 × 10⁻⁷ | 0.50 ± 0.03 | 4.0 ± 1.0 × 10⁻⁸ | 2.3 ± 1.0 × 10⁻⁸ |
| α₂βγδ₂₆α₆     | 3.2 ± 0.4 × 10⁻⁷ | 0.56 ± 0.03 | 2.3 ± 1.0 × 10⁻⁸ | 2.0 ± 0.7 × 10⁻⁸ |
| α₂βγδ₂₆α₆α₆   | 2.8 ± 0.4 × 10⁻⁷ | 0.55 ± 0.01 | 5.5 ± 1.1 × 10⁻⁸ | 2.7 ± 0.5 × 10⁻⁸ |
| ACh            |      |    |    |    |
| α₂βγδ          | 3.4 ± 0.2 × 10⁻⁷ | 0.56 ± 0.01 | 1.6 ± 0.4 × 10⁻⁷ | 5.7 ± 1.1 × 10⁻⁶ |
| α₂βγδ₂         | 9.1 ± 0.9 × 10⁻⁷ | 0.60 ± 0.03 | 2.8 ± 1.3 × 10⁻⁸ | 4.8 ± 1.8 × 10⁻⁸ |
| α₂βγδ₆       | 3.4 ± 0.8 × 10⁻⁷ | 0.47 ± 0.04 | 7.5 ± 1.8 × 10⁻⁷ | 3.6 ± 1.0 × 10⁻⁴ |
| α₂βγδ₆α₆     | 2.1 ± 0.5 × 10⁻⁵ | 0.50 ± 0.06 | 1.4 ± 0.4 × 10⁻⁷ | 6.6 ± 2.0 × 10⁻⁷ |
| α₂βγδ₆α₆α₆   | 2.8 ± 0.8 × 10⁻⁶ | 0.50 ± 0.06 | 2.3 ± 0.5 × 10⁻⁸ | 1.1 ± 0.2 × 10⁻³ |
| α₂βγδ₆α₆α₆   | 1.8 ± 0.2 × 10⁻⁴ | 0.61 ± 0.01 | 8.7 ± 1.0 × 10⁻⁹ | 2.4 ± 0.3 × 10⁻⁸ |
| α₂βγδ₆α₆α₆α₆ | 4.7 ± 0.4 × 10⁻⁴ | 0.67 ± 0.03 | 9.6 ± 1.5 × 10⁻⁵ | 1.8 ± 0.3 × 10⁻³ |
| α₂βγδ₆α₆α₆   | 4.5 ± 0.3 × 10⁻⁴ | 0.72 ± 0.03 | 10.0 ± 2.0 × 10⁻⁵ | 2.0 ± 0.4 × 10⁻³ |

Sucrose density gradient analysis was carried out to determine the size(s) of the receptors expressed in oocytes. To label surface nAChRs, intact oocytes were incubated with 125I-α-BgTx before isolation of oocyte membranes, and isolated membranes were reincubated in 125I-α-BgTx to label all sites made accessible after homogenization of the oocytes (total receptors). Membranes were extracted in 1% Triton X-100, and the sedimentation properties of these nAChRs were compared (Fig. 2) with 125I-α-BgTx-labeled native Torpedo nAChRs (monomer, 9.5 S; dimer, 13 S) (Fig. 2A). In the membranes isolated from oocyte homogenates, there were 3–5 times more total 125I-α-BgTx sites (Fig. 2, B–E, closed circles) than surface sites (Fig. 2, B–E, open circles). However, the major populations of both surface and total 125I-α-BgTx binding sites in oocyte membranes were pentameric nAChRs, as revealed by a characteristic large peak of 125I at 9.5 S for wild-type as well as mutant nAChRs (α₆γ, δ₆γ, ε₆γ, δ₆γε, Fig. 2, B–E). In some experiments (Figs. 2, B and D), total receptors in oocyte membranes also yielded a much smaller peak of 125I at 5.0 S, which has been shown previously to be subunit pairs of either αγ or αδ subunits (25). The peak at 1.7 S in all our experiments represented free proadifen produced only a modest (less than 3-fold) left shift of the ACh equilibrium binding function (data not shown), as it does for nAChRs in membranes from Torpedo electric organ (24), but in contrast to the 100-fold enhancement of ACh affinity seen for mouse muscle nAChR in the presence of proadifen (20).

Composition and Assembly of Subunits in Mutant nAChRs—To rule out the possibility that the perturbation of ACh binding resulted from nAChRs of altered subunit composition, we examined both the size and subunit composition of the wild-type and mutant nAChRs formed in our expression system. nAChR biosynthetic assembly intermediates can form high affinity binding sites for 125I-α-BgTx (α subunit alone) or agonists and competitive antagonists (αβ or αδ subunit pairs) (4). The binding studies described above were performed with membranes isolated from oocyte homogenates that contain both surface receptors and receptors from intracellular membranes. This internal pool may contain agonist binding sites that are very different than those found in the pentameric nAChRs, since the internal α-BgTx binding sites may include nAChR assembly intermediates. We therefore compared surface and internal receptors in oocyte membranes by examining both the size(s) of the 125I-α-BgTx binding components by sedimentation analysis and the subunit composition by biosynthetic labeling and immunoprecipitation.

Sucrose density gradient analysis was carried out to determine the size(s) of the receptors expressed in oocytes. To label surface nAChRs, intact oocytes were incubated with 125I-α-BgTx before isolation of oocyte membranes, and isolated membranes were reincubated in 125I-α-BgTx to label all sites made accessible after homogenization of the oocytes (total receptors). Membranes were extracted in 1% Triton X-100, and the sedimentation properties of these nAChRs were compared (Fig. 2) with 125I-α-BgTx-labeled native Torpedo nAChRs (monomer, 9.5 S; dimer, 13 S) (Fig. 2A). In the membranes isolated from oocyte homogenates, there were 3–5 times more total 125I-α-BgTx sites (Fig. 2, B–E, closed circles) than surface sites (Fig. 2, B–E, open circles). However, the major populations of both surface and total 125I-α-BgTx binding sites in oocyte membranes were pentameric nAChRs, as revealed by a characteristic large peak of 125I at 9.5 S for wild-type as well as mutant nAChRs (γ₆δ, δ₆γε, δ₆γ, Fig. 2, B–E). In some experiments (Figs. 2, B and D), total receptors in oocyte membranes also yielded a much smaller peak of 125I at 5.0 S, which has been shown previously to be subunit pairs of either αγ or αδ subunits (25). The peak at 1.7 S in all our experiments represented free proadifen produced only a modest (less than 3-fold) left shift of the ACh equilibrium binding function (data not shown), as it does for nAChRs in membranes from Torpedo electric organ (24), but in contrast to the 100-fold enhancement of ACh affinity seen for mouse muscle nAChR in the presence of proadifen (20).
Fig. 3. Biosynthetic labeling and immunoprecipitation analyses of the subunit composition of nAChRs containing γW55L and/or δW57L mutant subunits. 10–15 injected oocytes were incubated in ND96 (plus 50 μg/ml gentamicin) containing [35S]methionine/cysteine (0.1–0.5 mCi/ml) for 48 h. Surface (A) and total (B) receptors were labeled with nonradioactive α-β-gTx (see “Experimental Procedures”). Triton X-100 extracts containing either surface or total receptors labeled by α-β-gTx were incubated with affinity-purified rabbit antibody against α-β-gTx and immunoprecipitated with 1% Immunoprecipitin. Immunoprecipitates were extracted in sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis. Gels were stained with Coomassie Blue, destained, and prepared for fluorography (36-h exposure). For both surface (A) and total (B) receptors (1, αβγδ; 2, αβγδΔγ; 3, αβγδβγ; 4, αβγδγγ; 5, un.injected oocyte) γW55L and δW57L mutant subunits were expressed and assembled with other wild-type subunits as efficiently as seen for wild-type subunits.

Fig. 4. Electrophysiological recordings from wild-type and mutant nAChRs expressed in oocytes. A, inward currents evoked by 5 s applications of ACh to an oocyte expressing wild-type nAChRs. The oocyte was injected with diluted RNA (2.5 ng, a molar ratio of 2:1:1:1 αβγδ) to limit the current amplitudes at high ACh concentrations. The recordings were made 48 h after injection, and the oocyte membrane potential was held at −70 mV. B, concentration-dependent activation of γW55L mutant by ACh. The oocyte was injected with 100 ng RNA (2:1:1:1 αβγδ), which resulted in currents of similar magnitude as seen for wild-type nAChR after injection of lower amounts of RNA. C, dTC inhibition of ACh-induced currents for wild-type nAChR. Currents evoked by 3 μM ACh (3 s) from an oocyte injected with 10 ng of RNA were inhibited by preincubation with various concentrations of dTC. D, upon preincubation, dTC inhibited 3 μM ACh-induced currents from wild-type (WT) nAChRs (●) with an IC50 of 40 ± 4 nM (n = 0.9 ± 0.1). For γW55L mutant receptors (●) after preincubation, dTC inhibited 100 μM ACh-induced currents with an IC50 of 3.7 ± 0.6 μM (n = 0.9 ± 0.1). The data represent the mean ± S.D. of three measurements.

125I-α-BgTx. Thus, as for wild-type nAChRs, binding of 125I-α-BgTx to each of these mutants in membrane homogenates will reflect binding to assembled, pentameric nAChRs, and partial assembly cannot account for the altered ACh binding seen for the mutant nAChRs.

When the γ subunit was coexpressed with wild-type α and β subunits without the δ subunit (αβγδγγ, Fig. 2F), stable assembly of nAChR subunits was not observed in Triton X-100. This result, which was also seen for wild-type δ-less receptor (αβδγγ, data not shown), is consistent with previous observations (25, 26). For the mutant δ subunit coexpressed with wild-type α and β subunits without the γ subunit αβδγγγ, in addition to the 9.5 S peak of 125I, there was a prominent 5 S component in the total homogenate, as was seen for wild-type γ-less receptor (αββγγγ) (Figs. 2, G and H). These results indicated that partial assembly intermediates are not prominent for the mutant nAChRs containing all four subunits, but they may be more significant for wild-type or mutant nAChRs lacking the γ or δ subunit.

Subunit compositions of expressed wild-type and mutant nAChRs were characterized by immunoprecipitation of α-BgTx-labeled receptors (surface and total) extracted from oocyte homogenates after biosynthetic labeling with [35S]MeMet/Cys. Fig. 3 shows that for both surface (A) and total receptors (B), the presence of γW55L, δW57L, or both mutant subunits had no effect on subunit assembly, and the mutations had no effect on subunit glycosylation as judged by the mobilities of mutant compared with wild-type subunits. Furthermore, the mobilities of the subunits in nAChRs expressed in Xenopus oocytes were the same as the native Torpedo nAChR subunits (not shown). However, for nAChRs lacking the γ subunit, mutant δ subunit had the same mobility as wild type, but both had slightly higher mobility than δ subunits in nAChRs also containing the γ subunit. Similarly, omission of the δ subunit resulted in γ subunits of enhanced mobility (data not shown). γW55L and δW57L Alter nAChR Activation—We examined the effects of γW55L and δW57L on the activation of nAChRs expressed in oocytes using a two-electrode voltage clamp. When holding the oocyte membrane potential at −70 mV, there was a concentration-dependent activation of inward current upon 5-s application of ACh for wild-type (Fig. 4A) and mutant nAChRs (Fig. 4B). For wild-type nAChR, preincubation with dTC produced a dose-dependent inhibition of ACh currents characterized by an IC50 of 40 nM (Figs. 4, C and D), as expected when binding to its high affinity site results in functional antagonism. However, as reported previously (13) for nAChRs containing γW55L, dTC inhibited ACh currents only at higher concentrations (IC50 = 4 μM, Fig. 4D), despite the presence of the high affinity dTC binding site (Kd = 40 nM, Fig. 4A). Based upon the peak transient current observed for each ACh concentration, the ACh concentration for half-maximal response (Kap) for wild-type nAChRs was 20 ± 2 μM, whereas the Kap values for nAChRs containing γW55L or δW57L subunits were 170 ± 30 μM and 86 ± 2 μM, respectively (Fig. 5A). The double mutant receptor (αβγδγγ) was activated by ACh with a Kap of 340 ± 40 μM, about 15-fold higher than the wild-type receptor. The consequences of the leucine substitutions were clearly different from that seen for γ or δ subunit omission, since for γ-less (αβββγγ), Kap = 21 ± 4 μM and for δ-less (αβγγγγ), Kap = 14 ± 1 μM (not shown).

Fig. 5B shows a comparison of the number of 125I-α-BgTx binding sites on the oocyte surface and the current amplitudes for mutant nAChRs activated by saturating concentrations of ACh. nAChRs containing either γW55L, δW57L, or both mutant subunits were expressed at nearly the same levels as the wild-type nAChR, as indicated by the number of surface 125I-α-BgTx binding sites. In contrast, the maximal currents for the mutant nAChRs were much lower than for wild type. When equal amounts of subunit cRNAs were injected for wild-type and mutant nAChRs, the maximal currents for the δW57L...
and 0.6% that seen for the dose-response was fit by $K_d = 20 \pm 2 \mu M$ ($n = 6$ oocytes). For the $\gamma$W55L mutant (m, $\alpha_2\beta_3\gamma_0$), $K_d = 165 \pm 50 \mu M$ ($n = 4$ oocytes), and for the $\delta$W57L mutant (a, $\alpha_2\beta_3\gamma_0$), $K_d = 66 \pm 2 \mu M$ ($n = 3$ oocytes). For the double mutant (f, $\alpha_2\beta_3\gamma_0$), $K_d = 340 \pm 40 \mu M$ ($n = 4$ oocytes). Dose-response relations for wild-type receptors were determined for oocytes injected with 0.4 ng of subunit RNAs, whereas for mutant receptors, 10 ng were injected. The data represent the means ± S.D. B. oocytes were injected with 10 ng of subunit cRNAs. Mutant receptors were expressed on the oocyte surface at the same level as wild-type receptors as determined by $^{125}$I-a-BgTx binding (open bars, $n = 10$ oocytes). The maximal currents for the $\delta$W57L mutant were twice as large as the currents seen for wild-type receptors at 3 $\mu M$ ACh (batched bar), a concentration producing only 5% of maximal currents. The maximal currents (closed bars) were determined by saturating concentrations of ACh for $\gamma$W55L ($\alpha_2\beta_3\gamma_0$, 1.5 ± 0.3 $\mu M$, $n = 6$ oocytes) and for $\gamma$W55L/ $\delta$W57L ($\alpha_2\beta_3\gamma_0$, 0.16 ± 0.04 $\mu M$, $n = 6$ oocytes) mutants were only 6 and 0.6% that seen for the $\delta$W57L mutant ($\alpha_2\beta_3\gamma_0$). 25 ± 4 $\mu M$, $n = 5$ oocytes). The data represent the means ± S.D. C. Hill coefficients for ACh activation of wild-type and mutant nAChRs, determined from the concentration-response relationship at concentrations of ACh producing less than 20% of maximal currents. ACh-induced currents ($I_{\text{max}}$) recorded from wild-type (I), $\gamma$W55L (II), and $\delta$W57L (III) receptors were plotted logarithmically against the concentration of ACh, with each data point the mean ± S.D. of three recordings. The data for a single oocyte were fit by linear regression, and the slope of the line ($n_H$) for the wild-type receptor (I) was 1.85, with the same analysis for data from three oocytes characterized by a slope of 1.8 ± 0.1. For the $\gamma$W55L mutant (II), the slope for this representative experiment was 1.04, and the average slope from three experiments was 1.10 ± 0.05. The $\delta$W57L mutation, however, had no effect on the slope of the concentration-response relationship of ACh for receptor activation. The slope of the line for the $\delta$W57L mutant (I) presented here was 1.71, and the average value of the slope from three experiments was 1.66 ± 0.14.

The Hill coefficients ($n_H$) characterizing the ACh dose-response relations were estimated from log-log plots of current amplitude ($I$) versus [ACh] at concentrations of ACh producing currents less than 20% of maximal responses. This is a reliable method of determining $n_H$ for ACh responses without reference to the experimentally determined maximal currents, which are limited by desensitization and/or channel block. For the wild-type and $\delta$W57L mutant receptors, the concentration-response relationship had slope values ($n_H$) of 1.8 ± 0.1 and 1.7 ± 0.1, respectively (Fig. 5C). In contrast, the slope for the $\gamma$W55L mutant was one ($n_H = 1.1 ± 0.1$) (Fig. 5C).

dTC Potentiation of Agonist-induced Activation in $\gamma$W55L nAChRs—For the wild-type nAChR, upon preincubation, dTC acted as a competitive antagonist characterized by an IC$_{50}$ of 40 nM (Fig. 4D), and without preincubation, when coapplied with 3 $\mu M$ ACh for 5 s, dTC inhibited with an IC$_{50}$ of 250 nM (Fig. 6C). Similar results were obtained for nAChRs containing $\delta$W57L (data not shown). For nAChRs containing $\gamma$W55L, despite the fact that dTC binds with high affinity ($K_d = 40 \text{ nM}$) to one of the sites (Fig. 1A), upon preincubation, dTC inhibited ACh currents only at high concentrations (IC$_{50}$ = 4 $\mu M$, Fig. 4D), as reported by O’Leary et al. (13). We therefore examined in greater detail the interactions of dTC with nAChRs containing $\gamma$W55L. When applied alone, dTC produced no detectable whole cell currents ($<10 \text{nA}$, data not shown). However, we observed that at low concentrations dTC (10 nM to 1 $\mu M$) actually potentiated currents activated by ACh (10–100 $\mu M$) when both ligands were applied simultaneously to individual oocytes (Fig. 6). The magnitude of the potentiation was dependent on

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The Hill coefficients ($n_H$) for ACh responses without reference to the experimentally determined maximal currents, which are limited by desensitization and/or channel block. For the wild-type and $\delta$W57L mutant receptors, the concentration-response relationship had slope values ($n_H$) of 1.8 ± 0.1 and 1.7 ± 0.1, respectively (Fig. 5C). In contrast, the slope for the $\gamma$W55L mutant was one ($n_H = 1.1 ± 0.1$) (Fig. 5C).

dTC Potentiation of Agonist-induced Activation in $\gamma$W55L nAChRs—For the wild-type nAChR, upon preincubation, dTC acted as a competitive antagonist characterized by an IC$_{50}$ of 40 nM (Fig. 4D), and without preincubation, when coapplied with 3 $\mu M$ ACh for 5 s, dTC inhibited with an IC$_{50}$ of 250 nM (Fig. 6C). Similar results were obtained for nAChRs containing $\delta$W57L (data not shown). For nAChRs containing $\gamma$W55L, despite the fact that dTC binds with high affinity ($K_d = 40 \text{ nM}$) to one of the sites (Fig. 1A), upon preincubation, dTC inhibited ACh currents only at high concentrations (IC$_{50}$ = 4 $\mu M$, Fig. 4D), as reported by O’Leary et al. (13). We therefore examined in greater detail the interactions of dTC with nAChRs containing $\gamma$W55L. When applied alone, dTC produced no detectable whole cell currents ($<10 \text{nA}$, data not shown). However, we observed that at low concentrations dTC (10 nM to 1 $\mu M$) actually potentiated currents activated by ACh (10–100 $\mu M$) when both ligands were applied simultaneously to individual oocytes (Fig. 6). The magnitude of the potentiation was dependent on

\[ \delta \text{Trp-57 alters the activation of these channels by ACh.} \]
the concentration of both dTC and ACh (Fig. 6C), with the concentration dependence of potentiation by dTC for 10 μM ACh consistent with dTC binding to its high affinity binding site. The largest dTC potentiation was observed at low concentrations of ACh, and the magnitude of the potentiation decreased with higher concentrations of dTC. Concentrations above 10 μM dTC only inhibited currents activated by any concentration of ACh (Fig. 6C). For γW55L nAChRs preincubated with dTC, no potentiation was ever seen for responses to ACh at concentrations between 0.3 and 100 μM.

For the mutant receptor containing γW55L, potentiation by dTC was also observed for responses to two other agonists, carbamylcholine and suberyldicholine (Fig. 7). As for ACh, for these agonists the Hill coefficient (n_H) characterizing the dose-response relation was reduced from 1.6 for wild-type nAChRs to ~1 for the mutant receptor (Fig. 7A). dTC at concentrations between 10 nM and 1 μM produced a dose-dependent enhancement of responses for agonist concentrations producing submaximal responses. Higher dTC concentrations produced a progressive inhibition of the currents. For carbamylcholine, dTC enhanced currents as much as 3-fold (Fig. 7B), whereas for suberyldicholine, currents were increased by about 2-fold (Fig. 7C). For α_2β_3γδ, potentiation of agonist-induced currents was not limited to dTC, since metocurine, the 7′,12′-dimethoxy-2-methyl dTC analog, also potentiated activation of the mutant receptor by ACh (Fig. 7D). However, neither 13′-iodo-dTC nor two other competitive antagonists, pancuronium and gallamine, potentiated ACh responses (Fig. 7D). These results establish that when dTC (or metocurine) first binds to nAChRs containing γW55L, it does not act as an antagonist and, in contrast, acts as a coactivator or weak partial agonist, as evidenced by the potentiation of responses seen when agonists bind to the site at the ε-δ subunit interface.

**Influence of γW55L / δW57L on the Binding of Nicotinic Agonists and Antagonists**—We also examined the effects of the double mutation (α_2β_3γδ) on the binding equilibria of several agonists and antagonists to learn more about the effects of these substitutions on the binding of structurally diverse agonists and antagonists (Fig. 8). Most of the agonists tested, including tetramethylammonium, phenyltrimethylammonium, suberyldicholine, and epibatidine, inhibited 125I-α-BgTx binding to double mutant receptors with IC_{50} values that were ~50–500-fold larger than for the wild-type receptor. Thus, mutation of γTrp-55 and δTrp-57 influences the binding of the most nictinic agonists, even very small agonists like tetramethylammonium. It is interesting that the agonists with highest affinity for wild-type receptors were affected the most by the double mutation. One exception to the general pattern was nicotine, which bound to the wild-type and double mutant receptors with very similar affinity (Fig. 8F).
The effects of the double mutation (αβγδ) on the binding affinities of competitive antagonists are more diverse and complex than that seen for the agonists. The double mutation had no effect on the binding affinity of gallamine (Fig. 6G), similar to what was observed with dTC (Fig. 1). Pancuronium was bound with 10,000-fold selectivity by wild-type nAChRs (KiH = 3 nM, KJ = 20 μM), with binding at the high affinity site weakened by 100-fold in the double mutant and binding at the low affinity site weakened by less than 3-fold (Table II). The binding affinity of dihydro-β-erythroidine was decreased by about 10-fold at each site by the double mutation. The results of the binding experiments shown in Fig. 8 are summarized in Table II.

**DISCUSSION**

A wide body of evidence establishes that the two agonist binding sites in muscle-type nAChRs are positioned at the interfaces of α-γ and α-δ subunits, with amino acids contributed from three distinct regions of α subunit primary structure and at least three regions of γ (or δ) subunit (reviewed in Refs. 3, 8, and 9). We set out to study the functional contribution of two tryptophans, γTrp-55 and δTrp-57, at homologous positions of the γ and δ subunits. Tryptophans γTrp-55 and δTrp-57 are within or near the agonist/competitive antagonist binding sites, since they are the principle sites of specific photocorporation by [3H]dTC within those subunits (10), and γTrp-55 is the amino acid in the γ subunit specifically photolabeled by [3H]nicotine (11). In addition, they appeared likely to contribute to dTC binding affinity, because replacement of γTrp-55 by leucine resulted in a decrease of dTC potency as an inhibitor of ACh-induced currents for Torpedo nAChRs expressed in Xenopus oocytes (13). Although we confirmed the observation that for the γW55L mutant nAChR, the IC50 for dTC binding was increased 100-fold compared with wild type (Fig. 4), we were surprised to find that the mutation of either or both tryptophans had no effect on dTC equilibrium binding affinity, based upon the inhibition of binding of 125I-A-BgTx binding (Fig. 1B). The equilibrium binding function reflects both the affinity of binding to the desensitized state of the nAChR and the conformational equilibrium between resting and desensitized states. Before considering the effects of the substitutions, it is important to note several aspects of the observed binding to wild-type Torpedo nAChRs expressed in oocytes. The equilibrium binding of ACh by wild-type Torpedo nAChRs expressed in oocytes was well fit by a two-site model with an equal number of high and low affinity sites (Km = 55 nM, KJ = 3 μM). The parameters for ACh binding Torpedo αβγδ are similar to those seen for embryonic mouse nAChR expressed in oocytes (16), but they were quite different than those seen for ACh binding to Torpedo nAChR-Rich membranes, which was characterized by high affinity (Km = 25 nM) binding to a single site (Fig. 1B). We do not know the source of this difference, but it does not appear to result from a shift of the preexisting equilibrium between resting and desensitized states, since the desensitizing noncompetitive antagonist propridine had similar effects on ACh binding to either the native or expressed Torpedo nAChR. A noteworthy distinction between ACh interactions with Torpedo and embryonic mouse nAChR (αβγδ) is that our data indicate that in the Torpedo nAChR, ACh binds with higher affinity to the α-γ than to the α-δ site, whereas it binds with higher affinity at the α-δ site for the mouse nAChR (16, 27). The latter conclusion was based upon the observation that mouse subunit, but ACh binds with higher affinity at the α-δ site for the mouse nAChR (16, 27). The latter conclusion was based upon the observation that mouse αβδδ binds ACh with 15-fold higher affinity than αβγδ. For Torpedo nAChRs, ACh binds nonequivocally to the two sites even for receptors lacking the γ or δ subunit, but ACh binds with higher affinity to αβγδ than to αβδδ (Fig. 1B, Table I). For mouse nAChRs, preferential agonist binding to the α-δ site is not a general rule. For the nAChR containing an δ subunit in place of the γ subunit, ACh binds nonselectively at the two sites, whereas epibatidine binds with higher affinity at the α-γ (or α-ε) site than at the α-δ site, and for carbamylcholine the rank order is α-δ > α-ε > α-γ (28, 29).

Replacement of γTrp-55 by leucine had a much larger effect on ACh equilibrium binding than did replacement of δTrp-57 (Fig. 1 and Table I). Although the high and low affinity sites characteristic of the ACh equilibrium binding to wild-type nAChRs cannot be assigned unambiguously to binding at α-γ and α-δ sites, the observed binding by wild-type and mutant nAChRs is consistent with a simple interpretation if the high affinity ACh binding is at the α-γ site in wild-type nAChR, and that
TABLE II
Non-linear least squares fit of data in Figs. 1 and 8 according to Equations 1 and 2 (see “Experimental Procedures”). TMA, tetramethylammonium; PTA, phenyltrimethylammonium; Epi, epibatidine; Sub, suberidicholine; DHβE, dihydro-β-eucalyptol.

| Subunit | $K_L$ (nM) | $K_D$ (nM) | $n$ | $h$ |
|---------|------------|------------|-----|-----|
| TMA     | 11.1 ± 1.3 | 4.3 ± 0.7  | 0.12 ± 0.02 | 0.41 ± 0.03 |
| PTA     | 2.3 ± 0.4  | 0.6 ± 0.1  | 0.03 ± 0.01  | 0.08 ± 0.01  |
| Epi     | 0.01 ± 0.01 | 0.07 ± 0.03 | 0.17 ± 0.04  | 0.8 ± 0.1   |
| Sub     | 2.3 ± 0.3  | 0.5 ± 0.07 | 0.14 ± 0.10  | 0.8 ± 0.1   |
| DHβE    | 3.3 ± 0.8  | 1.4 ± 0.15 | 0.04 ± 0.01  | 0.09 ± 0.01  |

binding is 7,000–20,000-fold weaker in α6βγδ (K<sub>α</sub> = 360 μM) and α6βγδ<sup>m</sup> (K<sub>δ</sub> = 1 mm) but shifted less than 3-fold in α6βγδ<sup>e</sup> (K<sub>δ</sub> = 140 nM). ACh binds with low affinity (K<sub>α</sub> = 3 μM) at the α-δ site in wild-type nAChR, and that binding is preserved in α6βγ<sup>e</sup> (K<sub>δ</sub> = 0.8 μM) but weakened by 10–20 fold in α6βγ<sup>e</sup> (K<sub>δ</sub> = 66 μM) and α6βγ<sup>e</sup> (K<sub>δ</sub> = 23 μM). Thus the γW55L mutation disrupts ACh binding at the α-γ<sup>m</sup> site without causing a structural change that substantially perturbs ACh binding at the α-δ site. Within the α-γ<sup>m</sup> site, the change in structure must be limited, because the mutation does not alter DTC binding. If the selective disruption of ACh binding at equilibrium seen at the α-γ<sup>e</sup> site (not the α-δ site) in α6βγ<sup>e</sup> also occurs in the resting state, then the α-δ site will become the higher affinity site in the α6βγ<sup>e</sup> nAChR. Further studies at the level of single channel analysis will be required to determine the extent to which the leucine substitution alters the initial binding step or the conformational equilibria related to channel gating.

Since the leucine substitutions had no effect on the equilibrium binding of DTC, it was very surprising to observe for the γW55L nAChR that DTC binding to the high affinity (α-γ) site no longer acted as an antagonist. Rather, inhibition of ACh responses was seen only when DTC was bound to the α-δ site, and DTC actually acted as a partial agonist or coagonist when it bound to the mutated α-γ site. These observations appear to suggest paradoxically that the γW55L substitution converted DTC from an antagonist into a partial agonist without having any effect on the energetics of its binding. However, an alternative explanation is that the mutation alters ACh binding only, with a secondary consequence that this mutation reveals the functional consequences of DTC occupancy of its high affinity site when ACh is binding to the α-δ site. Two lines of reasoning support this interpretation. First, the experimental results can be accounted for by a three-state allosteric model (resting, open, desensitized) that assumes that ACh binding to the α-δ site as well as DTC binding to both sites is unaltered between wild-type and γW55L mutant AChRs. By adjusting parameters only for ACh binding at the α-γ site in the three states, it is possible to account for the observed shift of ACh $K_a$ and the Hill coefficient, the equilibrium binding of ACh and DTC to wild-type and mutant nAChRs, and the observed concentration dependence of DTC potentiation of the ACh responses in the mutant nAChR. Second, although we have not found experimental conditions where DTC acts as a coactivator for wild-type Torpedo nAChR, DTC and metocurine (but not pancuronium) act as weak partial agonists potentiating the responses seen at low ACh concentrations for rat and mouse muscle embryonic nAChRs (α2βδγ) (30–33). In contrast, they all act as competitive antagonists at adult nAChRs (α2βδδ). Despite the difference in pharmacology, DTC binds with the same high affinity to the mouse α-γ<sup>e</sup> site as to the α-δ site, but as we discussed above, ACh binds with higher affinity to the α-δ site than to the α-γ<sup>e</sup> site in the embryonic nAChR, whereas it binds nonselectively to the α-ε and α-δ sites (29).

Replacement of γTrp-55 by leucine weakens ACh equilibrium binding at the α-γ<sup>e</sup> site by 7,000-fold, a decrease of affinity substantially larger than the ~20–500-fold shift of affinity seen for substitutions of the conserved tyrosines within the α subunit binding loops (34) or for charge-neutralizing mutations (αD152Q, γD174N, δD180N) in mouse nAChR (16, 18, 35). For nAChRs containing γD174N/δD180N as well as for nAChRs containing αD152Q the mutations appear to affect ligand binding directly rather than the conformational equilibria between resting and desensitized states (18). However, for the simplest
agonist, tetramethylammonium, equilibrium binding is weakened by 100-fold for the γW55L mutant, for γD174N (16), and also by substitutions at αTyr-93, αTyr-190, and αTyr-198 (34). It is unlikely that tetramethylammonium is simultaneously in contact with all of these side chains.

Although the presence of γTrp-55 within the agonist binding site was identified on the basis of its photolabeling by [3H]dTC, leucine substitution has no effect on dTC binding affinity. It is likely that replacement by other amino acids will alter dTC binding affinity, because even the leucine substitution weakens the binding of the competitive antagonists dihydro-β-erythroidine and pancuronium by 10- and 70-fold, respectively. In addition, substitution of the corresponding tryptophan in homooligomeric α7 nAChRs (α7Trp54) by histidine weakened dihydro-β-erythroidine binding by 10-fold (36).

Our results establish the importance of γTrp-55 for agonist binding and channel gating, but they do not establish that ACh interacts directly with this tryptophan. In studies of mutant Torpedo nAChRs containing cysteines within the binding site, there was no evidence that γW55C was accessible for reaction with cationic methythiosulfonates, whereas an adjacent position (γE57C) as well as an α93C and an α198C were all accessible for modification (37). This result, in conjunction with the fact that γTrp-55 is photolabeled by [3H]dTC and [3H]nicotine, suggests that γTrp-55 may be within a hydrophobic subdomain of the binding site.

Substitutions at positions equivalent to nAChR γTrp-55 also have important functional consequences for other members of the superfamily of ligand-gated ion channels related to the nAChRs. For α1β2γ2 γ-aminobutyric acid type A receptors, replacement of α1Phe-64 by leucine results in a 200-fold decrease in dTC binding affinity, because even the leucine substitution weakens the binding of the competitive antagonists dihydro-β-erythroidine and pancuronium by 10- and 70-fold, respectively. In addition, substitution of the corresponding tryptophan in homooligomeric α7 nAChRs (α7Trp54) by histidine weakened dihydro-β-erythroidine binding by 10-fold (36).

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Contributions of *Torpedo* Nicotinic Acetylcholine Receptor γTrp-55 and δTrp-57 to Agonist and Competitive Antagonist Function

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