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Molecular Determinants of (+)-Tubocurarine Binding at Recombinant 5-Hydroxytryptamine$_{3A}$ Receptor Subunits

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ABSTRACT

The 5-hydroxytryptamine type 3 (5-HT$_3$) receptor is a transmitter-gated ion channel mediating neuronal excitation. The receptor native to neurons, or as a homopentameric assembly of 5-HT$_{3A}$ receptor subunits, displays a species-dependent pharmacology exemplified by a 1800-fold difference in the potency of (+)-tubocurarine [(+)-Tc] as an antagonist of the current response mediated by mouse and human receptor orthologs. Here, we attempt to identify amino acid residues involved in binding (+)-Tc by use of chimeric and mutant 5-HT$_{3A}$ subunits of mouse and human expressed in *Xenopus laevis* oocytes. Replacement of the entire extracellular N-terminal domain of the mouse 5-HT$_{3A}$ (m5-HT$_{3A}$) subunit by that of the human ortholog and vice versa exchanged the differential potency of (+)-Tc, demonstrating the ligand binding site to be contained wholly within this region. Mutagenesis of multiple amino acid residues within a putative binding domain that exchanged nonconserved residues between mouse and human receptors shifted the apparent affinity of (+)-Tc in a reciprocal manner. The magnitude of the shift increased with the number of residues (3, 5, or 7) exchanged, with septuple mutations of m5-HT$_{3A}$ and human 5-HT$_{3A}$ subunits producing a 161-fold decrease and 53-fold increase in the apparent affinity of (+)-Tc, respectively. The effect of point mutations was generally modest, the exception being m5-HT$_{3A}$ D206E, which produced a 9-fold decrease in apparent affinity. We conclude that multiple amino acids within a binding loop of human and mouse 5-HT$_{3A}$ subunits influence the potency of (+)-Tc.

The 5-hydroxytryptamine type 3 (5-HT$_3$) receptor is a transmitter-gated, cation-selective ion channel containing five transmembrane-spanning glycoprotein subunits (Derkach et al., 1989; Boess et al., 1995). A 5-HT$_3$ subunit, termed 5-HT$_{3A}$, has been identified by expression cloning from a murine hydridoma cell line cDNA library (Maricq et al., 1991). Subsequent homology screening has allowed the isolation of splice variants and 5-HT$_{3A}$ subunit orthologs from human (Belelli et al., 1995; Miyake et al., 1995), rat (Isenberg et al., 1993; Miyake et al., 1995), and guinea pig (Lankiewicz et al., 1998) sources. Most recently, a novel 5-HT$_{3B}$ subunit, capable of forming hetero-oligomeric complexes with the 5-HT$_{3A}$ subunit, has been identified (Davies et al., 1999).

Despite their highly conserved structure, 5-HT$_{3A}$ subunit orthologs display distinctive pharmacological profiles that closely reflect the interspecies variation in ligand binding described for neuronal 5-HT$_3$ receptors (Peters et al., 1997). A striking example is provided by the nicotinic acetylcholine receptor (AChR) antagonist (+)-tubocurarine [(+)-Tc], which binds to native (pK_i = 6.7–7.3; Bonhaus et al., 1993) or recombinant receptors (pK_i = 6.7–7.1; Bonhaus et al., 1995; Yan et al., 1999) of mouse origin (m5-HT$_{3A}$) with an affinity comparable with that found for the high- affinity site formed at the interfaces of the α/γ subunits of the *Torpedo* nicotinic AChR (Pedersen and Cohen, 1990) and the α/γ and α/ϵ subunits of the mammalian skeletal muscle nicotinic AChR (Sine, 1993; Papineni and Pedersen, 1997; Bren and Sine, 1997). In contrast, (+)-Tc demonstrates much lower affinity at the human recombinant (h5-HT$_{3A}$; pK_i = 4.4; Hope et al., 1996) or native (pK_i = 4.8; Bufton et al., 1993) receptor isoforms.

Knowledge of the structural determinants of ligand binding at 5-HT$_3$ receptors is limited. That the extracellular N-terminal domain of the receptor imparts ligand binding specificity is evident from studies on a chimeric construct of the N-terminal domain of the nicotinic AChR α7 subunit and the residual sequence of the m5-HT$_{3A}$ subunit (Eiselé et al., 1993). The chimera displayed nicotinic receptor pharmacology, but the ion channel properties of the m5-HT$_{3A}$ receptor. Oxidation of tryptophan residues within the 5-HT$_3$ receptor reduces the binding of the selective antagonist [^{3}H]zacopride in a manner that can be prevented by preincubation with some, but not all, 5-HT$_3$ receptor ligands (Miquel et al., 1991). For the m5-HT$_{3A}$ subunit, the replacement of individ-

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ABBREVIATIONS: AChR, acetylcholine receptor; 5-HT, 5-hydroxytryptamine; PCR, polymerase chain reaction; (+)-Tc, (+)-tubocurarine.

ual tryptophan residues within the N-terminal domain by either Tyr and/or Ser causes, with the exception of Trp-67, loss of ligand recognition and function (Spier et al., 1997). Replacement of Trp-67 by Tyr or Phe decreased the affinity of (+)-Tc, but also other ligands, such as granisetron (Spier et al., 1997; Yan et al., 1999). Interestingly, Trp occupies an homologous location in nicotinic AChR α7, γ, and δ subunits (i.e., α7Trp-54, γTrp-55, and δTrp-57) and participates in the binding of (+)-Tc (O'Leary et al., 1994; Corringer et al., 1995). However, Trp-67 and adjacent residues are conserved in all 5-HT_{3A} subunit orthologs, indicating that additional residues must be responsible for the differential potency of (+)-Tc. Similarly, although mutation of Glu-106 of the m5-HT_{3A} subunit produces differing effects upon the binding of several ligands (Boess et al., 1997), conservation of this residue and flanking sequences argues against involvement in the differential affinity of (+)-Tc.

The window of selectivity between the binding of (+)-Tc at high (i.e., α/γ or α/ϵ) and low (i.e., α/δ) affinity subunit interfaces of the muscle nicotinic AChR has, via the construction of chimeras and site-directed mutations, revealed residues that participate in (+)-Tc binding (Pedersen and Cohen, 1990; Sine, 1993; Papineni and Pedersen, 1997; Bren and Sine, 1997). Here, we exploit the differential affinity of (+)-Tc at m5-HT_{3A} and h5-HT_{3A} receptors to identify residues that might influence binding. We elected to quantify antagonism by (+)-Tc using an electrophysiological assay of wild-type and mutant 5-HT_{3A} subunits expressed in *Xenopus laevis* oocytes because of the robust difference (i.e., ~1,850-fold) in the potency of the antagonist at human and mouse 5-HT_{3A} receptors in this system (Hope et al., 1993; Belelli et al., 1995; Peters et al., 1997). Subunit chimeras wherein the N-terminal domains of human and mouse 5-HT_{3A} receptor subunits were exchanged reciprocally were constructed to verify that differences in the potency of (+)-Tc are due wholly to binding in the extracellular N-terminal region. By the construction of mutant receptors, we demonstrate the reciprocal exchange of small numbers of residues between human and mouse receptors to produce opposite effects upon the potency of (+)-Tc.

Experimental Procedures

Materials. (+)-Tubocurarine hydrochloride and 5-HT creatine sulfate complex were purchased from Sigma Chemical Co. (Poole, Dorset, UK). Oligonucleotides were synthesized by Cruachem Ltd. (Glasgow, Strathclyde, UK).

Construction of Chimeric 5-HT_3 Receptor Subunits and Mutagenesis. Two chimeric 5-HT_3 receptor subunits containing human and mouse 5-HT_{3A} sequences were constructed using a polymerase chain reaction (PCR)-based approach (Yon and Fried, 1989). The chimeric receptor subunit h218m 5-HT_{3A}, where the number denotes the position of the residue immediately amino terminal to the chimeric junction, comprised the extracellular N-terminal domain of the h5-HT_{3A} subunit and the remaining sequence of the m5-HT_{3A} ortholog. The 5′ and 3′ ends of the h218m 5-HT_{3A} chimera were defined by the oligonucleotides H1 (CCGGAATTCCGGGGCCACGAGAGGCAG) and M1 (CCGCTCGAGAAGATATCATAGCATTTTTTATT) containing *Eco*RI and *Xho*I restriction sites (underlined), respectively. The chimeric junction was defined by a single large oligonucleotide C1 (ATGTGGTCATCCGCCGGCGGCCTTTATTCTATGCAGTCAG). Conversely, the amino- and carboxy-terminal components of m223h 5-HT_{3A} were derived from the corresponding regions of m5-HT_{3A} and h5-HT_{3A}, respectively. The 5′ and 3′ ends of m223h 5-HT_{3A} were defined by the oligonucleotides M2 (CCGGCTCGAGACATCTGGGAAGCTTGCCAT) and H2 (CCGGAATTCCAAAGTCCC) embodying an *Xho*I or *Eco*RI site. The chimeric junction was defined by oligonucleotide C2 (ACGTGATCATCCGCCGGAGGCCCCTCTTCTATGTGGTCAG). Both chimeric 5-HT_{3A} subunits were amplified using a 30-cycle PCR (denaturing, 95°C, 30 s; annealing, 65°C, 30 s; and extension, 75°C, 4 min). Each PCR contained 10 ng of m5-HT_{3A} and h5-HT_{3A} cDNAs, 10 mM KCl 10, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-Cl (pH 8.8), 2 mM $MgSO_4$, 0.1% Triton X-100, 100 μg ml^{-1} BSA, 200 μM each dNTP and 2.5 U of plaque-forming unit polymerase. In both cases, the outer (5′ and 3′) primers were present at a concentration of 1 μM, while the central oligonucleotide (C1 or C2) was present at 0.01 μM. h218m 5-HT_{3A} and m223h 5-HT_{3A} were cloned into pcDNA1amp (In Vitrogen BV, NV Leek, The Netherlands) and Bluescript SK+ (Stratagene Ltd., Cambridge, UK) respectively, before expression in *Xenopus laevis* oocytes.

For site-directed mutagenesis, m5-HT_{3A} and h5-HT_{3A} were cloned into the eukaryotic expression vector pcDNA1amp, under the control of the cytomegalovirus promoter. Single-stranded template cDNAs were synthesized from the M13 origin of replication and mutations were generated using standard procedures. Oligonucleotides coding for the mutated sequences were used to prime individual mutagenesis reactions. In addition, each mutagenic oligonucleotide incorporated an additional silent mutation encoding a novel restriction site, which allowed for rapid screening for mutated 5-HT_{3A} cDNAs by restriction analysis.

The fidelity of all chimera and mutagenesis reactions was confirmed by standard dideoxynucleotide sequencing (*fmol* DNA Sequencing System; Promega, Southhampton, UK) of the entire coding sequences of the 5-HT_{3A} cDNAs.

Expression of Chimeras, Mutant Receptors, and Electrophysiological Analysis. *Xenopus laevis* ooctyes were isolated and enzymatically defolliculated as previously described (Hope et al., 1993). cDNA (20 nl; 5–250 ng μl^{-1}) encoding chimeric or mutant 5-HT_{3A} receptor subunits was injected into the nucleus of Stage V-VI oocytes, which were subsequently stored individually at 19–20°C for 2 to 10 days in 96-well microtiter plates in 200 μl of Barth's solution (composition 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 1 mM $MgSO_4$, 0.5 mM $CaCl_2$, 0.5 mM $Ca(NO_3)_2$, and 15 mM HEPES, pH 7.5) supplemented with gentamicin (100 μg ml^{-1}).

Electrical recordings were made under conventional two- electrode voltage-clamp using either an Axoclamp 2A or GeneClamp 500 amplifier (Axon Instruments, Foster City, CA). Recording and current passing electrodes were filled with 3 M KCl and 3 M CsCl, respectively, and had resistances in the range 0.6 to 2.0 MΩ when measured in standard extracellular solution (composition: 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 1 mM $MgSO_4$, 0.5 mM $CaCl_2$, and HEPES 10, pH 7.5). Oocytes were held in a Perspex (ICI Acrylics, Darwen, UK) chamber of 0.5 ml volume and constantly superfused with extracellular solution at a rate of 8 to 10 ml min^{-1}. All agonist and antagonist compounds were applied via the superfusate. Antagonists were preapplied for a period of 1 min before simultaneous application with agonist for an additional 20 to 60 s. Currents evoked by 5-HT (at EC_{50}, see below) were recorded onto digital audiotape using a Biologic DAT recorder (Biologic Science Instruments, Claix, France) and displayed upon a chart recorder. All recordings were conducted at ambient temperature (18–23°C).

In all experiments examining antagonism of agonist evoked currents by (+)-tubocurarine, 5-HT was applied at the EC_{50} determined for the chimeric or mutant receptor under evaluation. Agonist concentration response curves were iteratively fitted (Fig P. V6; BioSoft, Cambridge, UK) with the Hill equation:

$$\frac{I}{I_{max}} = \frac{[A]^{nH}}{[A]^{nH} + [EC_{50}]^{nH}}$$

where I is the peak inward current evoked by agonist at concentration A, I_{max} is the maximal inward current evoked by a saturating concentration of agonist, EC_{50} is the concentration of agonist induc-

ing a half-maximal current response, and nH is the Hill coefficient. An equation of the same form was used to analyze the concentration dependence of antagonist induced blockade of the 5-HT-evoked response, i.e.

$$\frac{I}{I_{max}} = \frac{[B]^{-n}}{[B]^{-n} + [IC_{50}]^{-n}}$$

where B is antagonist concentration, IC_{50} is the concentration of antagonist producing half-maximal inhibition of the control response to 5-HT, n is the interaction coefficient, and I and I_{max} are as previously defined. IC_{50} and EC_{50} values are expressed as the mean and S.E. as derived from individual fitted parameters (see Tables 1 and 2).

Results

Mouse and Human 5-HT$_{3A}$ Subunit Chimeras. Previous studies examining the antagonist potency of (+)-Tc at h5-HT$_{3A}$ and m5-HT$_{3A}$ receptors expressed in *Xenopus laevis* oocytes yielded IC_{50} values of 2.6 μM and 1.4 nM, respectively (Hope et al., 1993; Belelli et al., 1995). To confirm that this large differential is due exclusively to differences in primary amino acid sequence residing within the extracellular N-terminal domain preceding the first transmembrane span, the chimeras m223h 5-HT$_{3A}$ and h218m 5-HT$_{3A}$ were constructed and examined for sensitivity to block by (+)-Tc. At a holding potential of −60 mV, both chimeric constructs mediated large inward current responses to 5-HT applied at a half-maximally effective concentration (EC_{50}; Fig. 1). As anticipated from the close similarity in the EC_{50} values for 5-HT at wild-type m5-HT$_{3A}$ and h5-HT$_{3A}$ subunits (Hope et al., 1993; Belelli et al., 1995), the apparent affinity of 5-HT at either chimera was unaltered (Table 1). In contrast, the concentration of (+)-Tc required to reduce the control response to 5-HT by 50% (i.e., IC_{50}) at chimera m233h 5-HT$_{3A}$ was reduced by over 2200-fold relative to the wild-type h5-HT$_{3A}$ subunit (Table 1), such that the concentration-inhibition curve shifted leftward to superimpose upon that obtained for (+)-Tc acting at the wild-type m5-HT$_{3A}$ subunit (Fig. 1). The converse result was obtained for the h218m 5-HT$_{3A}$ subunit chimera, where the IC_{50} for (+)-Tc was increased by over 1300-fold in comparison to the m5-HT$_{3A}$ subunit to yield an inhibition curve that approximated closely to that found for (+)-Tc at the wild-type h5-HT$_{3A}$ subunit. These results indicate that the structural determinants of the discriminatory potency of (+)-Tc are located entirely within the N-terminal domain.

Multiple Point Mutant m5-HT$_{3A}$ and h5-HT$_{3A}$ Subunits. Sequence alignment of 5-HT$_{3A}$ subunit orthologs (Fig. 2) reveals a cluster of nonconserved residues contained within a domain homologous to ligand binding "loop" 3 (also termed "C") of certain nicotinic AChR (Galzi and Changeux, 1995), γ-aminobutyric acid type A (Smith and Olsen, 1995), and strychnine-sensitive glycine receptor subunits (Rajendra et al., 1997). In view of the fact that (+)-Tc is known to photoaffinity label aromatic residues within loop 3 of nicotinic AChR α-subunits, which stabilize the binding of the antagonist (Sine et al., 1994; Chiara and Cohen, 1997), we focused upon the homologous region of m5-HT$_{3A}$ and h5-HT$_{3A}$ in subsequent mutagenesis experiments.

Initially, three consecutive nonconserved residues within the putative loop 3 region were selected for mutagenesis. The triplet mutation m5-HT$_{3A}$ I205 M/D206E/I207S (i.e., mutant 1) shifted the antagonist potency of (+)-Tc toward that observed for the h5-HT$_{3A}$ subunit, producing, on average, a 20-fold increase in IC_{50} relative to that observed for the wild-type m5-HT$_{3A}$ subunit (Table 1; Figs. 3 and 5). The reciprocal mutation h5-HT$_{3A}$ M200I/E201D/S202I (i.e., mutant 2), while producing a qualitatively opposite affect, enhanced the potency of (+)-Tc by only ~5-fold (Table 1 and Figs. 4 and 5).

The additional mutations m5-HT$_{3A}$ Q199Y/K201R and h5-HT$_{3A}$ Y194Q/R196K, producing quintuple mutants 3 (m5-HT$_{3A}$ Q199Y/K201R/I205M/D206E/I207S) and 4 (h5-HT$_{3A}$ Y194Q/R196K/M200I/E201D/S202I), produced further shifts in the IC_{50} for (+)-Tc. For mutant 3, this amounted to a 54-fold decrease and for mutant 4, a 26-fold increase relative to mouse and human wild-type subunits, respectively (Table 1 and Figs. 3, 4, and 5). Finally, mutation of the two remaining nonconserved residues within the putative loop 3 region (i.e., m5-HT$_{3A}$ S210Y/I219V and h5-HT$_{3A}$ Y205S/V214I), yielding the septuple mutants 5 and 6, caused a further modest increment (~3-fold) and decrement (~2-fold) of (+)-Tc IC_{50} values relative to mutants 3 and 4, respectively (Table 1 and Figs. 3, 4, and 5).

As summarized in Table 1, the EC_{50} for 5-HT (1.4–5.8 μM) and the Hill coefficient of concentration-effect relationship (nH; 1.5–2.7) were similar across wild-type, chimeric, and multiple point mutant receptors, suggesting that the gross

TABLE 1

Summary of potencies of (+)-Tc and 5-HT acting at mouse and human wild-type and chimeric 5-HT$_{3A}$ receptor subunits

IC_{50} values for (+)-Tc were calculated from a single-site model fitted to data as concentration of antagonist required to reduce inward current response evoked by 5-HT at EC_{50} by 50%.

Receptor	(+)-Tc IC_{50} (mean ± S.E.)	*n*	5-HT EC_{50} (mean ± S.E.)	nH
	nM		*μM*	
m5-HT$_{3A}$ wild type[a]	1.40 ± 0.15	0.90 ± 0.10	2.30 ± 0.10	2.20 ± 0.20
h5-HT$_{3A}$ wild type[b]	2550.00 ± 150	1.00 ± 0.10	3.10 ± 0.10	1.94 ± 0.14
m223h 5-HT$_{3A}$	1.16 ± 0.07	1.06 ± 0.07	2.56 ± 0.26	1.99 ± 0.22
h218m 5-HT$_{3A}$	1870.00 ± 510	1.28 ± 0.09	2.84 ± 0.40	1.94 ± 0.08
Mutant 1	27.50 ± 9.2	1.53 ± 0.15	4.66 ± 0.49	1.53 ± 0.07
Mutant 2	486.00 ± 76	1.50 ± 0.09	1.70 ± 0.13	2.23 ± 0.18
Mutant 3	76.00 ± 12.5	1.39 ± 0.18	5.84 ± 0.55	1.48 ± 0.09
Mutant 4	99.50 ± 4.26	1.17 ± 0.17	1.44 ± 0.80	2.33 ± 0.33
Mutant 5	228.00 ± 18	1.15 ± 0.15	4.79 ± 0.35	1.87 ± 0.04
Mutant 6	49.40 ± 3.4	1.37 ± 0.27	2.24 ± 0.40	1.75 ± 0.46

Mutant 1, m5-HT$_{3A}$ I205M/D206E/I207S; mutant 2, h5-HT$_{3A}$ M200I/E201D/S202I; mutant 3, m5-HT$_{3A}$ Q199Y/K201R/I205M/D206E/I207S; mutant 4, h5-HT$_{3A}$ Y194Q/R196K/M200I/E201D/S202I; mutant 5, m5-HT$_{3A}$ Q199Y/K201R/I205M/D206E/I207S/S210Y/I219V; mutant 6, h5-HT$_{3A}$ Y194Q/R196K/M200I/E201D/S202I/Y205S/V214I.

[a] Data from Hope et al. (1993).

[b] Data from Belelli et al. (1995).

structural and allosteric properties of the expressed protein were unaltered. We therefore suggest that the observed changes in the IC_{50} of (+)-Tc are due to the antagonist interacting, either directly or indirectly, with multiple residues located within the loop 3 region. In subsequent studies with single point mutants, we attempted to identify the residue(s) that exert greatest impact upon the potency of (+)-Tc.

Single Point Mutant m5-HT_{3A} Subunits. Table 2 summarizes the IC_{50} values for (+)-Tc obtained when the seven nonidentical amino acids within the putative loop 3 of the m5-HT_{3A} subunit were individually mutated to the homologous residues of the h5-HT_{3A} species ortholog. The mutations m5-HT_{3A}K201R, I205 M, I207S, and I219V were associated with IC_{50} values for (+)-Tc that were essentially indistinguishable from that found for the wild-type m5-HT_{3A} subunit. Very modest increases in IC_{50} (~2.5–3.0-fold) were obtained for m5-HT_{3A}Q199Y and S210Y, whereas a substantial increment (~9-fold) was found for the D206E mutant.

Discussion

Domains contributing to the ligand binding sites of nicotinic AChRs were initially identified by affinity labeling using derivatives of agonist and antagonist compounds. Such studies provided a valuable framework for subsequent molecular biological approaches employing chimeric and point mutant receptor subunits (reviewed in Galzi and Changeux, 1995). In the absence of similar information, we exploited the species-dependent pharmacology of 5-HT_{3A} receptor subunits and sequence comparisons between subunits as an attempt to delineate components of the ligand binding site.

(+)-Tc is a structurally rigid antagonist that displays a large difference in potency across 5-HT_{3A} subunit orthologs (Peters et al., 1997). One potential advantage in evaluating this ligand is the existence of comparative information concerning specific amino acid residues that influence the binding of (+)-Tc to the interfaces formed between muscle nicotinic AChR subunits (Pedersen and Cohen, 1990; O'Leary et al., 1994; Sine, 1993; Bren and Sine, 1997; Chiara and Cohen, 1997). In the latter case, the binding of (+)-Tc involves specific domains provided both by the α and non-α subunits, which have been termed the "principal" and "complementary" components of the site respectively (Bertrand and Changeux, 1995). The structure activity relationships for curariform antagonists at *Torpedo* and mouse nAChR α/γ subunit interfaces and the m5-HT_{3A} subunit are broadly similar (Pedersen and Papineni, 1995; Papineni and Pedersen, 1997; Yan et al., 1998), suggesting that the binding of (+)-Tc at the 5-HT_3 receptor may also involve principal and complementary components provided, in this instance, by the opposite faces of adjacent, structurally identical, 5-HT_{3A} subunits. The results of the present study implicating loop 3 residues ("principal component") in the binding of (+)-Tc when combined with data demonstrating an influence of

TABLE 2

Summary of potency of (+)-Tc acting at wild-type mouse and point mutant 5-HT_{3A} receptor subunits

IC_{50} values for (+)-Tc were calculated as in Table 1.

Receptor	(+)-Tc IC_{50} (mean ± S.E.)	*n*
	nM	
m5-HT_{3A} (wild type)[a]	1.4 ± 0.15	0.9 ± 0.1
m5-HT_{3A}Q199Y	3.2 ± 0.80	1.0 ± 0.1
m5-HT_{3A}K201R	1.4 ± 0.30	1.3 ± 0.1
m5-HT_{3A}I205M	2.3 ± 0.10	0.9 ± 0.1
m5-HT_{3A}D206E	13.6 ± 3.60	1.0 ± 0.1
m5-HT_{3A}I207S	0.7 ± 0.20	1.1 ± 0.1
m5-HT_{3A}S210Y	4.5 ± 0.60	1.3 ± 0.1
m5-HT_{3A}I219V	1.9 ± 0.20	1.4 ± 0.5

[a] Data from Hope et al. (1993).

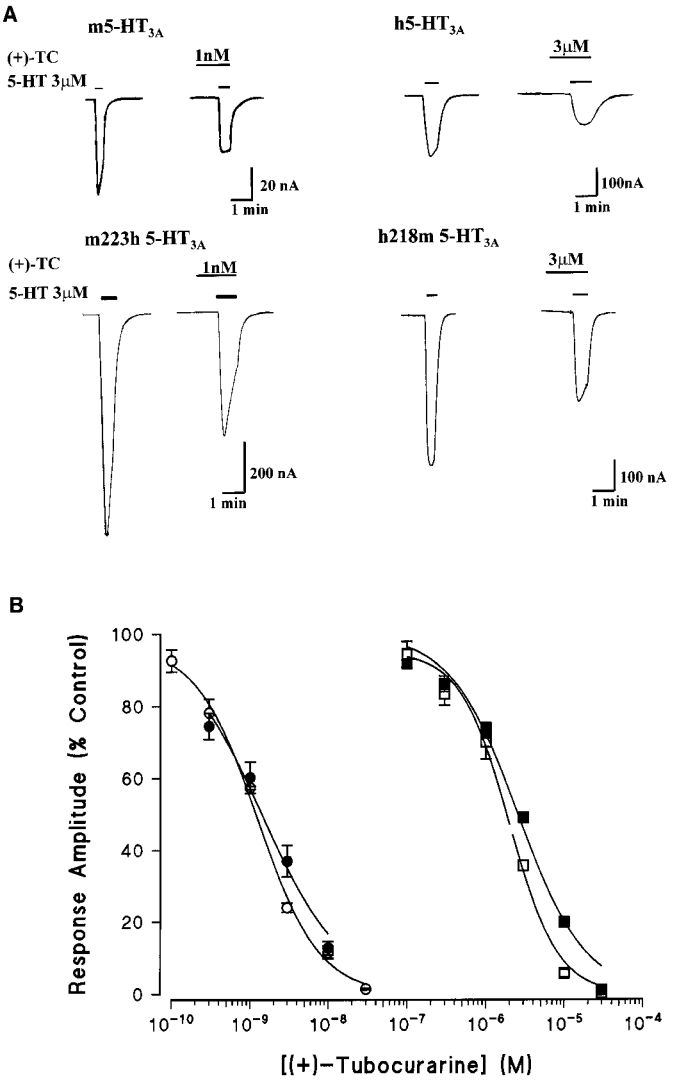

Fig. 1. Antagonist potency of (+)-Tc at wild-type human and mouse 5-HT_{3A} receptor subunits in comparison to m223h 5-HT_{3A} and h218m 5-HT_{3A} subunit chimeras. A, traces illustrating potency of (+)-Tc in inhibiting currents evoked by equieffective (i.e., EC_{50}) concentrations of 5-HT at wild-type human and mouse 5-HT_{3A} receptor subunits and m223h 5-HT_{3A} and h218m 5-HT_{3A} subunit chimeras. (+)-Tc (1 nM) inhibits ~50% of current evoked by 3 μM 5-HT both at wild-type m5HT_{3A} receptor subunit and m223h 5-HT_{3A} subunit chimera. In contrast, 3 μM (+)-Tc is required to produce ~50% inhibition of 5-HT (3 μM)-evoked current both at h5HT_{3A} receptor subunit and h218m 5-HT_{3A} subunit chimera. B, graph illustrates relationship between concentration of (+)-Tc in medium (abscissa, log scale) and peak amplitude of inward current response evoked by 5-HT at EC_{50} as a percentage of control response to 5-HT (ordinate, linear scale) for wild-type m5-HT_{3A} (●), wild-type h5-HT_{3A} (■), m223 h 5-HT_{3A} (○), and h218m 5-HT_{3A} (□) subunits. Data points were fitted with equation described in *Experimental Procedures* to yield the following parameters: m5-HT_{3A}, IC_{50} = 1.4 nM, n = 0.9; h5-HT_{3A}, IC_{50} = 2.55 μM, n = 1.00; m223h 5-HT_{3A}, IC_{50} = 1.16 nM, n = 1.06; h218m 5-HT_{3A}, IC_{50} = 1.87 μM, n = 1.28. Each point represents mean ± S. E. of experiments conducted upon a minimum of three oocytes. Data for m5-HT_{3A} and h5-HT_{3A} were taken from Hope et al. (1993) and Belelli et al. (1995), respectively.

Trp-67 ("complementary component") upon antagonist binding (Yan et al., 1999) support such a scheme.

Studies on native and recombinant 5-HT$_3$ receptors indicate that (+)-Tc acts in a manner consistent with competitive antagonism (Higashi and Nishi, 1982; Maricq et al., 1991; Newberry et al., 1991; Hope et al., 1993; Yan et al., 1998). Indeed, as assessed by computational chemistry, there is considerable structural congruence between (+)-Tc, 5-HT, and several 5-HT$_3$ receptor-selective agonists (Aprison et al., 1996). However, high concentrations of (+)-Tc insurmountably antagonize electrical responses mediated by 5-HT$_3$ receptors endogenous to rabbit (Higashi and Nishi, 1982) and guinea pig (Newberry et al., 1991) neurons. By analogy to the muscle nAChR, such an action could potentially be due to open channel blockade by (+)-Tc (Colquhoun et al., 1979). Thus, it was important to confirm that the differential potency of (+)-Tc at mouse and human 5-HT$_{3A}$ subunit orthologs is entirely due to differences in primary amino acid sequence within the extracellular N-terminal domain. That this is so is indicated by the results obtained with the chimeras m223h 5-HT$_{3A}$ and h218m 5-HT$_{3A}$, where the reciprocal exchange of the N-terminal domain was shown to entirely account for the species dependent pharmacology of (+)-Tc.

We studied the domain homologous to loop 3 (or C) of nAChR α subunits for several reasons. First, sequence alignment of the four orthologs of the 5-HT$_{3A}$ subunit currently isolated identify this region as containing a particularly high incidence of unconserved residues relative to the remainder of the extracellular N-terminal domain (Fig. 2). Secondy, a chimeric construct of the human and guinea pig 5-HT$_{3A}$ subunit orthologs reveals this region to strongly contribute to a differential potency of 1-phenylbiguanide, a 5-HT$_3$ receptor-selective agonist, at the wild-type subunits. Third, a tyrosine residue (Tyr-198) within loop 3 of nAChR α subunits that is photoaffinity labeled by (+)-Tc (Chiara and Cohen, 1997) and that constitutes an important element of the principal binding component (Sine, 1993; O'Leary et al., 1994), is conserved in the 5-HT$_{3A}$ subunit (Fig. 2 and see below).

The substitution of amino acid residues from m5-HT$_{3A}$ into h5-HT$_{3A}$ sequence and vice versa caused qualitatively opposite changes in the potency of (+)-Tc. By contrast, only very modest effects upon the agonist potency of 5-HT were observed, militating against a nonspecific effect of the mutations upon receptor structure or function. The progressively larger shift in the IC$_{50}$ of (+)-Tc associated with the triplet, quintuple, and septuple amino acid substitutions in loop 3 (Table 1) indicates that multiple nonconserved residues contribute to the differential potency of the antagonist at mouse and human 5-HT$_{3A}$ receptor orthologs. Furthermore, it is clear that additional residues located elsewhere within the

Human 5-HT$_{3A}$	P-**YFR**EFS-**MES**SN**Y**YAEMKFYV**V**IRRR	218
Mouse 5-HT$_{3A(b)}$	P-**QFK**EFS-**IDI**SN**S**YAEMKFYV**I**IRRR	223
Rat 5-HT$_{3A(b)}$	T-KFQEFS-IETSNSYAEMKFYVVIRRR	223
Guinea-pig 5-HT$_{3A(b)}$	T-EFLEFSDRESRGSFAEMKFYVVIRRR	224
Mouse nAChR α1	GWKHWVFYSCCPTTPYLDITYHFVMQRL	210
Torpedo nAChR α1	GWKHWVYYTCCPDTPYLDITYHFIMQRI	210
	^ ^^ ^	

Fig. 2. Sequence alignment of species homologs of 5-HT$_{3A}$ subunit and nicotinic acetylcholine receptor α subunit of mouse and *Torpedo* over domain corresponding to loop 3 of nicotinic receptor. Residues that are not conserved between human and mouse 5-HT$_{3A}$ subunit orthologs are highlighted in **bold**. Residues corresponding to Tyr- 190, Cys-192, Cys-193, and Tyr-198 of nicotinic α1 sequence are indicated (^).

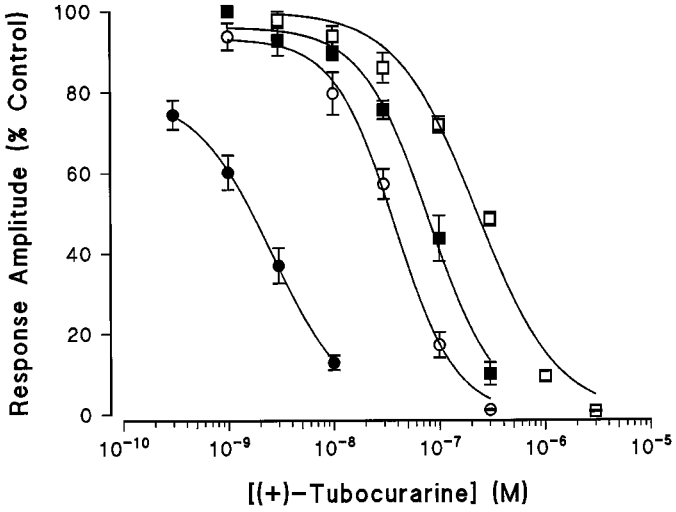

Fig. 3. Antagonist potency of (+)-Tc at the wild-type m5-HT$_{3A}$ receptor subunit in comparison with m5-HT$_{3A}$ subunits bearing triple, quintuple, or septuple amino acid mutations. Graph depicts relationship between concentration of (+)-Tc in medium (abscissa, log scale) and peak amplitude of inward current response evoked by 5-HT at EC$_{50}$ as a percentage of control response to 5-HT (ordinate, linear scale) for wild-type m5-HT$_{3A}$ (●), mutant 1 (i.e., m5-HT$_{3A}$ I205M/D206E/I207S; ○), mutant 3 (i.e., m5-HT$_{3A}$ Q199Y/K201R/I205M/D206E/I207S; ■) and mutant 5 (i.e., m5-HT$_{3A}$ Q199Y/K201R/I205M/D206E/I207S/S210Y/I219V; □) receptor subunits. Data points were fitted with equation described in *Experimental Procedures* to yield the following parameters: m5-HT$_{3A}$, IC$_{50}$ = 1.4 nM, n = 0.9; mutant 1, IC$_{50}$ = 27.5 nM, n = 1.53; mutant 3, IC$_{50}$ = 76 nM, n = 1.39; and mutant 5, IC$_{50}$ = 228 nM, n = 1.15. Each point represents mean ± S.E. of experiments conducted on a minimum of three oocytes. Data for m5-HT$_{3A}$ were taken from Hope et al. (1993).

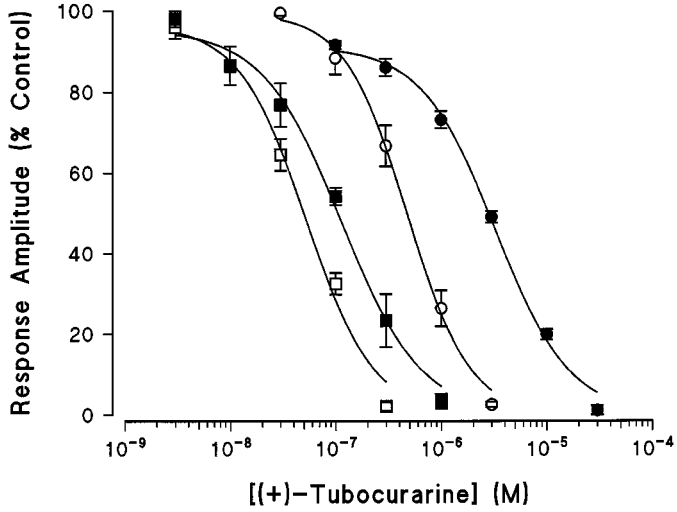

Fig. 4. Antagonist potency of (+)-Tc at the wild-type h5-HT$_{3A}$ receptor subunit in comparison with h5-HT$_{3A}$ subunits bearing triple, quintuple, or septuple amino acid mutations. Graph depicts relationship between concentration of (+)-Tc in medium (abscissa, log scale) and peak amplitude of inward current response evoked by 5-HT at EC$_{50}$ as a percentage of control response to 5-HT (ordinate, linear scale) for wild-type h5-HT$_{3A}$ (●), mutant 2 (i.e., h5-HT$_{3A}$ M200I/E201D/S202I; ○), mutant 4 (i.e., h5-HT$_{3A}$ Y194Q/R196K/M200I/E201D/S202I; ■) and mutant 6 (i.e., m5-HT$_{3A}$ Y194Q/R196K/M200I/E201D/S202I/Y205S/V214I; □) receptor subunits. Data points were fitted with equation described in *Experimental Procedures* to yield the following parameters: h5-HT$_{3A}$, IC$_{50}$ = 2.55 μM, n = 1.0; mutant 2, IC$_{50}$ = 486 nM, n = 1.53; mutant 4, IC$_{50}$ = 99.5 nM, n = 1.17; and mutant 6, IC$_{50}$ = 49.4 nM, n = 1.37. Each point represents mean ± S.E. of experiments conducted on a minimum of three oocytes. Data for h5-HT$_{3A}$ were taken from Belelli et al. (1995).

N-terminal domain must also be involved, because the exchange of all seven nonconserved loop 3 amino acids between the subunit orthologs is insufficient to completely convert the IC_{50} for (+)-Tc to that of either the human (cf. mutant 5) or mouse (cf. mutant 6) 5-HT_{3A} subunit. Moreover, such unidentified residues appear to contribute unequally to the binding of (+)-Tc in the two subunit orthologs as evidenced by the consistently smaller impact upon the IC_{50} of (+)-Tc when homologous residues from the mouse are grafted into the human subunit versus the converse exchange (compare mutants 1, 3, and 5 with mutants 2, 4, and 6). In this respect, domains of the 5-HT_{3A} subunit that are homologous to the complementary binding sites for (+)-Tc presented by vertebrate muscle nicotinic AChR γ/ϵ (high affinity) and δ (low affinity) subunits are of interest, particularly because Trp-67, which is homologous to α7Trp-54, γTrp-55, and δTrp-57, participates in the binding of (+)-Tc (Spier et al., 1997; Yan et al., 1999). However, at loci corresponding to the critical residues identified in the nicotinic AChR subunits [i.e., fetal receptor: γIle-116/δVal-118; γTyr-117/δThr-119 (Sine, 1993); γSer-161/δLys-163; adult receptor: ϵIle-58/δHis-60; and ϵAsp-59/δAla-61 (Bren and Sine, 1997)] the primary sequence across 5-HT_{3A} subunit orthologs is invariant, or shows only a conservative substitution (i.e., Phe/Tyr) that, in any event, does not correlate with the apparent affinity of (+)-Tc.

The analysis of the contribution of individual residues suggests that, in all but one instance, interactions with (+)-Tc are likely to be indirect. Thus, the mutations m5-HT_{3A} Q199Y, K201R, I205M, I207S, S210Y, or I219V produce, at most, a 3-fold change in the blocking potency of (+)-Tc. Several of these exchanges conserve gross physicochemical properties of the residue such as positive charge (K201R) and aliphatic (I219V) character, whereas others are associated with the incorporation of aromatic groups and a concomitant increase in side chain volume (Q199Y and S210Y). It is noteworthy that there is little conservation of these residues across the four orthologs of 5-HT_{3A} subunit thus far identified (Fig. 2). In contrast, in the case of the solitary mutation producing a substantial increase in the IC_{50} of (+)-Tc (i.e., m5-HT_{3A} D206E) Glu is the aligned residue in nonmouse 5-HT_{3A} subunits, all of which demonstrate reduced affinity toward (+)-Tc. Interestingly, m5-HT_{3A} Asp-206 aligns with Cys-193 of the nicotinic AChR α-subunit which, along with Cys-192, Tyr-190, and Tyr198, contributes to the loop 3 component of the principal nicotinic binding site (Galzi and Changeux, 1995; Bertrand and Changeux, 1995).

There is considerable evidence that both Tyr-190 and Tyr-198 of the muscle nicotinic AChR α-subunit act to stabilize the binding of the curariform antagonist dimethyl-*d*-tubocurarine, principally through quaterary ammonium-aromatic interactions (O'Leary et al., 1994; Sine et al., 1994). An homologous tyrosine residue common to human, mouse, and rat 5-HT_{3A} subunits, or phenylalanine in the guinea pig sequence (Fig. 2), may play a similar role. However, a serine residue conserved across all 5-HT_{3A} subunit orthologs aligns with Tyr-190 (Fig. 2). Mutagenesis of Tyr-190 to Ser in the nicotinic AChR subunits is associated with a pronounced reduction in the affinity of dimethyl-*d*-tubocurarine and the virtual abolition of α/γ versus α/δ interface selectivity (Sine et al., 1994). It is conceivable that this substitution contributes to the low affinity of (+)-Tc for the human 5-HT_{3A} receptor and that the multiple amino acid differences between human

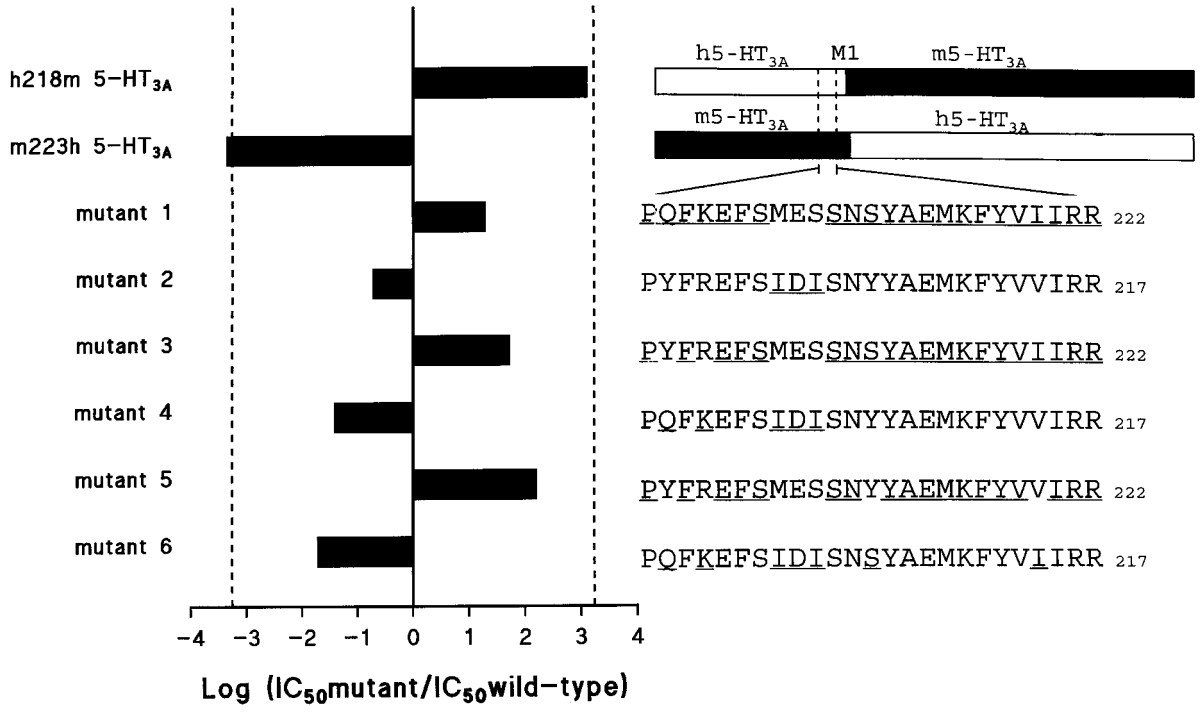

Fig. 5. Determinants of species-dependent antagonist potency of (+)-Tc at human (h) and mouse (m) 5-HT_{3A} receptor subunits. Left, potency of (+)-Tc as an antagonist of inward current response to 5-HT at EC_{50} is expressed as log of ratio of the IC_{50} at chimeric and mutant receptors divided by IC_{50} at appropriate wild-type receptor. Vertical dashed lines indicate log IC_{50} ratios m5-HT_{3A}/h5-HT_{3A} and h5-HT_{3A}/m5-HT_{3A}. Right, schematic representation of chimeras h218m 5-HT_{3A} and m223h 5-HT_{3A} (mouse sequence shaded) and position of loop 3 domain. Loop 3 residues exchanged between h5-HT_{3A} and m5-HT_{3A} subunits are detailed in text, with sequence corresponding to m5-HT_{3A} underlined. Bar diagram was constructed from data presented in Table 1.

and mouse sequences within loop 3 compensate for the presence of serine in the mouse subunit, perhaps by allowing the adjacent phenylalanine residue to assume an orientation that permits interaction with (+)-Tc. Overall, the present data are compatible with a scheme wherein loop 3 residues collectively determine the shape of an element of the ligand pocket for (+)-Tc with m5-HT$_{3A}$ Asp-206, perhaps making direct contact with the ligand. The additional methylene group in the side chain of Glu versus Asp may place the negatively charged carboxylic acid group in a less favorable orientation for interaction with (+)-Tc.

A precedent for a diffuse influence of loop 3 residues upon ligand binding derives from a recent study of the chimeric α7-5-HT$_{3A}$ subunit (Corringer et al., 1998). In the latter, the exchange of five residues from an agonist binding domain of the α4- nicotinic subunit into the α7-5-HT$_{3A}$ chimera selectively enhanced the apparent affinity of ACh by 30-fold, abolishing the difference in potency between the latter and nicotine to confer a pharmacological phenotype typical of the $\alpha4\beta2$-nicotinic receptor. In common with the results of the present study, the mutation of individual residues revealed only one that exerted a substantial (i.e., 7-fold) influence upon the apparent affinity of ACh. Interestingly, several of the residues exerting a subtle effect upon the apparent affinity of ACh are homologous to amino acids within mouse and human 5-HT$_{3A}$ subunits that contribute to the differential potency of (+)-Tc.

References

Aprison MH, Galvez-Ruano E and Lipkowicz KB (1996) Comparison of binding mechanisms at cholinergic, serotonergic, glycinergic and GABAergic receptors. *J Neurosci Res* **43:**127–136.

Belelli D, Balcarek JM, Hope AG, Peters JA, Lambert JJ and Blackburn TP (1995) Cloning and functional characterization of a human 5-hydroxytryptamine type 3A$_S$ receptor subunit. *Mol Pharmacol* **48:**1054–1062.

Bertrand D and Changeux J-P (1995) Nicotinic receptor: An allosteric protein specialized for intercellular communication. *Semin Neurosci* **7:**75–90.

Boess FG, Beroukim R and Martin IL (1995) Ultrastructure of the 5-hydroxytryptamine$_3$ receptor. *J Neurochem* **64:**1401–1405.

Boess FG, Steward LJ, Steele JA, Liu D, Reid J, Glencourse TA and Martin, IL (1997) Analysis of the ligand binding site of the 5-HT$_3$ receptor using site directed mutagenesis: Importance of glutamate 106. *Neuropharmacology* **36:**637–647.

Bonhaus DW, Stefanich E, Loury DN, Hsu SAO, Eglen RM and Wong, EHF (1995) Allosteric interactions among agonists and antagonists at 5-hydroxytryptamine$_3$ receptors. *J Neurochem* **65:**104–110.

Bonhaus DW, Wong EHF, Stefanich E, Kunysz EA and Eglen RM (1993) Pharmacological characterization of 5-hydroxytryptamine$_3$ receptors in murine brain and ileum using the novel radioligand [^{3}H]RS-422358–197: Evidence for receptor heterogeneity *J Neurochem* **61:**1927–1932.

Bren N and Sine SM (1997) Identification of residues in the adult nicotinic acetylcholine receptor that confer selectivity for curariform antagonists. *J Biol Chem* **272:**30793–30798.

Bufton KE, Steward LJ, Barber PC and Barnes NM (1993) Distribution and characterization of the [^{3}H]granisetron-labelled 5-HT$_3$ receptor in the human forebrain. *Neuropharmacology* **32:**1325–1331.

Chiara DC and Cohen JB (1997) Identification of amino acids contributing to high and low affinity *d*-tubocurarine sites in the *Torpedo* nicotinic acetylcholine receptor. *J Biol Chem* **272:**32940–32950.

Colquhoun D, Dreyer F and Sheridan RE (1979) The actions of tubocurarine at the frog neuromuscular junction. *J Physiol (London)* **293:**247–284.

Corringer P-J, Bertrand S, Bohler S, Edelstein SJ, Changeux J-P and Bertrand D (1998) Critical elements determining diversity in agonist binding and desensitization of neuronal nicotinic acetylcholine receptors. *J Neurosci* **18:**648–657.

Corringer P-J, Galzi J-L, Eiselé J-L, Bertrand S, Changeux, J-P and Bertrand D (1995) Identification of a new component of the agonist binding site of the nicotinic α7 homooligomeric receptor. *J Biol Chem* **270:**11749–11752.

Davies PA, Pistis M, Hanna MC, Peters JA, Lambert JJ, Hales TG and Kirkness EF (1999) The 5-HT$_{3B}$ subunit is a major determinant of serotonin receptor function. *Nature* **397:**359–363.

Derkach VA, Surprenant A and North RA (1989) 5-HT$_3$ receptors are membrane ion channels. *Nature* **339:**706–709.

Eiselé J-L, Bertrand S, Galzi J-L, Devillers-Thiéry A, Changeux J-P and Bertrand D (1993) Chimaeric nicotinic-serotonergic receptor combines distinct ligand binding and channel specificities. *Nature* **366:**479–483.

Galzi J-L and Changeux J-P (1995) Neuronal nicotinic receptors: Molecular organization and regulations. *Neuropharmacology* **34:**563–582.

Higashi H and Nishi S (1982) 5-Hydroxytryptamine receptors on visceral primary afferent neurones of rabbit nodose ganglia. *J Physiol (London)* **323:**543–567.

Hope AG, Downie DL, Sutherland L, Lambert JJ, Peters JA and Burchell B (1993) Cloning and functional expression of an apparent splice variant of the murine 5-HT$_3$ receptor A subunit. *Eur J Pharmacol* **245:**187–192.

Hope AG, Peters JA, Brown AM, Lambert JJ and Blackburn TP (1996) Characterization of a human 5-hydroxytryptamine$_3$ receptor type A (h5-HT$_3$R-A$_S$) subunit stably expressed in HEK 293 cells. *Br J Pharmacol* **118:**1237–1245.

Isenberg KE, Ukhun IA, Holstad SG, Jafri S, Uchiro U, Zorumski CF and Yang J (1993) Partial cDNA cloning and NGF regulation of a rat 5-HT$_3$ receptor subunit. *Neuroreport* **5:**121–124.

Lankiewicz S, Lobitz N, Wetzel CHR, Rupprecht R, Gisselmann G and Hatt H (1998) Molecular cloning, functional expression, and pharmacological characterization of 5-hydroxytryptamine$_3$ receptor cDNA and its splice variants from guinea-pig. *Mol Pharmacol* **53:**202–212.

Maricq AV, Peterson AS, Brake AJ, Myers RM and Julius D (1991) Primary structure and functional expression of the 5-HT$_3$ receptor, a serotonin-gated ion channel. *Science* **254:**432–437.

Miquel MC, Emerit MB, Gozlan H and Hamon M (1991) Involvement of tryptophan residue(s) in the specific binding of agonists/antagonists to 5-HT$_3$ receptors in NG 108–15 clonal cells. *Biochem Pharmacol* **42:**1456–1461.

Miyake A, Mochizuki S, Takemoto Y and Akusawa, S (1995) Molecular cloning of human 5-hydroxytryptamine$_3$ receptor: Heterogeneity in distribution and function among species. *Mol Pharmacol* **48:**407–416.

Newberry NR, Cheshire SH and Gilbert MJ (1991) Evidence that the 5-HT$_3$ receptors of the rat, mouse and guinea-pig superior cervical ganglion may be different. *Br J Pharmacol* **102:**615–620.

O'Leary ME, Filatov GN and White MM (1994) Characterization of *d*-tubocurarine binding site of *Torpedo* acetylcholine receptor. *Am J Physiol* **266:**C648–C653.

Papineni RVL and Pedersen SE (1997) Interaction of *d*-tubocuarine analogues with the mouse nicotinic acetylcholine receptor. *J Biol Chem* **272:**24891–24898.

Pedersen SE and Cohen JB (1990) *d*-Tubocurarine binding sites are located at α-γ and α-δ subunit interfaces of the nicotinic acetylcholine receptor. *Proc Natl Acad Sci USA* **87:**2785–2789.

Pedersen SE and Papineni RVL (1995) Interaction of *d*-tubocurarine analogs with the *Torpedo* nicotinic acetylcholine receptor. *J Biol Chem* **270:**31141–31150.

Peters JA, Hope AG, Sutherland L and Lambert JJ (1997) Recombinant 5-hydroxytryptamine receptors, in *Recombinant Cell Surface Receptors: Focal Point for Therapeutic Intervention* (Brown MJ ed) pp 119–154, RG Landes, Austin, TX.

Rajendra S, Lynch JW and Schofield PR (1997) The glycine receptor. *Pharmacol Ther* **73:**121–146.

Sine SM (1993) Molecular dissection of subunit interfaces in the acetylcholine receptor: Identification of residues that determine curare sensitivity. *Proc Natl Acad Sci USA* **90:**9436–9440.

Sine SM, Quiram P, Pananikolaou F, Kreienkamp H-J and Taylor P (1994) Conserved tyrosines in the α subunit of the nicotinic acetylcholine receptor stabilize quaternary ammonium groups of agonist and curariform antagonists. *J Biol Chem* **269:**8808–8816.

Smith GB and Olsen RW (1995) Functional domains of GABA$_A$ receptors. *Trends Pharmacol Sci* **16:**162–168.

Spier AD, Chu J and Lummis SCR (1997) The role of tryptophan residues in the 5-HT$_3$ receptor binding site. *Soc Neurosci Abs* **23:**59.10.

Yan D, Pedersen SE and White MW (1998) Interaction of *d*-tubocurarine analogs with the 5HT$_3$ receptor. *Neuropharmacology* **37:**251–257.

Yan D, Schulte MK, Bloom K and White MM (1999) Structural features of the ligand-binding domain of the serotonin 5-HT$_3$ receptor. *J Biol Chem* **274:**5537–5541

Yon J and Fried M (1989) Precise gene fusion by PCR. *Nucleic Acids Res* **17:**4895.

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