Structural Features of the Ligand-binding Domain of the Serotonin 5HT$_3$ Receptor*

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The nicotinic acetylcholine receptor (AChR) and the serotonin type 3 receptor (5HT$_3$R) are members of the ligand-gated ion channel gene family. Both receptors are inhibited by nanomolar concentrations of *d*-tubocurarine (curare) in a competitive fashion. Chemical labeling studies on the AChR have identified tryptophan residues on the γ (γTrp-55) and δ (δTrp-57) subunits that interact with curare. Comparison of the sequences of these two subunits with the 5HT$_3$R shows that a tryptophan residue is found in the homologous position in the 5HT$_3$R (Trp-89), suggesting that this residue may be involved in curare-5HT$_3$R interactions. Site-directed mutagenesis at position Trp-89 markedly reduces the affinity of the 5HT$_3$R for the antagonists curare and granisetron but has little effect on the affinity for the agonist serotonin. To further examine the role of this region of the receptor in ligand-receptor interactions, alanine-scanning mutagenesis analysis of the region centered on Trp-89 (Thr-85 to Trp-94) was carried out, and the ligand binding properties of the mutant receptors were determined. Within this region of the receptor, curare affinity is reduced by substitution only at Trp-89, whereas serotonin affinity is reduced only by substitution at Arg-91. On the other hand, granisetron affinity is reduced by substitutions at Trp-89, Arg-91, and Tyr-93. This differential effect of substitutions on ligand affinity suggests that different ligands may have different points of interaction within the ligand-binding pocket. In addition, the every-other-residue periodicity of the effects on granisetron affinity strongly suggests that this region of the ligand-binding site of the 5HT$_3$R (and by inference, other members of the ligand-gated ion channel family) is in a β-strand conformation.

The serotonin type 3 receptor (5HT$_3$R)[1] is a member of a superfamily of ligand-gated ion channels, which includes the muscle and neuronal nicotinic acetylcholine receptor (AChR), the glycine receptor, and the γ-aminobutyric acid type A receptor (1–3). Like the other members of the gene superfamily, the 5HT$_3$R exhibits a large degree of sequence similarity, and thus presumably structural homology, with the AChR (4). Chimeras containing the amino-terminal domain of the α7 neuronal AChR and the carboxyl-terminal domain of the 5HT$_3$R form functional ligand-gated channels with ligand specificities characteristic of AChR and permeability properties of 5HT$_3$Rs (5). These experiments suggest that the AChR and 5HT$_3$R have quite similar structures and signal transduction mechanisms.

A large number of studies have been conducted to elucidate the structure of the ligand-binding site for AChRs (6, 7); however, very little is known about the interactions between ligands and the 5HT$_3$R. A common competitive antagonist of the AChR and 5HT$_3$R is *d*-tubocurarine (curare), which inhibits both receptors at nanomolar concentrations (8, 9). If both receptors are similar in structure, it seems reasonable to assume that similar regions of the receptors may be involved in curare binding. Affinity labeling studies using the *Torpedo* electroplax AChR have identified two tryptophan residues, one on the γ (γTrp-55) and the other on the δ (δTrp-57) subunit, as being involved in curare-AChR interactions (10). Mutagenesis data are consistent with γTrp-55 contributing to the high affinity curare-binding site (11), which is known to exist at the α-γ subunit interface (12, 13).

Comparison of the deduced amino acid sequences of this region of a number of members of the gene superfamily shows that tryptophan is found in this position in the AChR γ (γTrp-55) and δ (δTrp-57) subunits and the 5HT$_3$R (position Trp-89) but not most other receptors (Fig. 1). In support of the notion that Trp-89 may contribute to the curare-binding site of 5HT$_3$Rs, chemical modification studies have suggested that one or more tryptophan residues contribute to the binding of a number of 5HT$_3$R ligands (14). To determine the role of Trp-89 in ligand-5HT$_3$R interactions, we have carried out an alanine scan mutational analysis (15) of the region around Trp-89. We have identified several residues in this region that play a role in ligand-receptor interactions and have further identified residues that may make differential contributions to agonist and antagonist binding. Furthermore, the periodicity of the effects of the mutations on ligand affinity suggests that this particular region of the ligand-binding domain is in a β-strand conformation.

EXPERIMENTAL PROCEDURES

Isolation of 5HT$_3$R cDNA and Mutagenesis—A pair of oligonucleotides (GCCTACTACTGTCTCCATTGA and CTCCCACTCGCCCTGATTTAT) derived from the published sequence of the original 5HT$_3$R cDNA (4) was used to amplify a 460-base pair fragment from RNA isolated from the murine neuroblastoma line NIE-115 using reverse transcription-polymerase chain reaction (RNA polymerase chain reaction kit, P/E/Express). The fragment was then used to screen a plasmid cDNA library made from N1E-115 mRNA. A full-length cDNA clone corresponding to the 5HT$_3$RA$_s$ form (16) was isolated and subcloned into vector pCI (Promega, Madison, WI). Site-directed mutagenesis was performed using the QuickChange system (Stratagene, La Jolla, CA). All mutants were verified through sequence analysis. Because the amino terminus of the mature protein is unknown, the amino acid numbering system used here includes the signal sequence and has the initial methionine as position 1.

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[1] The abbreviations used are: 5HT$_3$R, serotonin type 3 receptor; AChR, nicotinic acetylcholine receptor.

Secondary Structure Analysis—Protein secondary structure analysis was carried out using the Chou-Fasman (17) and Garnier-Robson (18) algorithms contained in a commercially available software package (Lasergene, DNAStar, Inc., Madison, WI).

Transfection—Cultures of the tsA201 cell line, a derivative of the widely used HEK 293 cell line, were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 units/ml streptomycin. Cultures at 30–40% confluence were transfected with 20 μg of 5HT$_3$R cDNA/100-mm dish using the calcium phosphate technique. After 12-h exposure to the DNA/calcium phosphate solution, the medium was replaced with fresh medium, and the cells were allowed to grow for another 24–36 h prior to use. Maximal expression was obtained 36–72 h after transfection.

Ligand Binding Assays—Transfected cells were scraped from the dishes, washed once with phosphate-buffered saline and resuspended and homogenized in 2.5 ml of 154 mM NaCl, 50 mM Tris-HCl, pH 7.4, per 100-mm dish. The homogenate was then used in binding assays or frozen until needed. We observed no change in either ligand affinity or B_{max} values after freezing.

Membranes were incubated for 2 h at 37 °C in a total volume of 0.5 ml of 154 mM NaCl, 50 mM Tris-HCl, pH 7.4, containing the appropriate concentrations of the competing ligand and radioligand ([^{3}H]granisetron; NEN Life Science Products, 85 Ci/mmol). Binding was terminated by rapid vacuum filtration onto GF/B filters that had been pretreated in 0.2% polyethyleneimine in 50 mM Tris-HCl, pH 7.4, and the filters were washed with cold 50 mM Tris-HCl, pH 7.4. Nonspecific binding was defined as that binding not displaced by 100 μM *m*-chlorophenyl biguanide. K_d values for [^{3}H]granisetron were determined by fitting the saturation binding data to Equation 1 using a Levenberg-Marquardt algorithm in a commercially available software package (Igor Pro, WaveMetrics, Oswego, OR),

$$B = \frac{B_{max}}{1 + (K_d/[L])} \quad \text{(Eq. 1)}$$

where B is the amount of [^{3}H]granisetron specifically bound at concentration [L], B_{max} is the maximal binding at saturation, and K_d is the dissociation constant. IC$_{50}$ values for various inhibitors were determined by fitting the data to Equation 2,

$$\theta = \left(1 + \frac{[I]}{IC_{50}}\right)^{-1} \quad \text{(Eq. 2)}$$

where θ is the fractional amount of [^{3}H]granisetron bound in the presence of the inhibitor at concentration [I] compared with that in the absence of inhibitor and IC$_{50}$ is the concentration of inhibitor at which $\theta = 0.5$. K_i values were calculated from the IC$_{50}$ values using the Cheng-Prusoff relation (19) (Equation 3),

$$K_i = \frac{IC_{50}}{1 + ([L]/K_d)} \quad \text{(Eq. 3)}$$

where [L] is the concentration of [^{3}H]granisetron used to determine the IC$_{50}$ value in the experiment and K_d is the dissociation constant for [^{3}H]granisetron.

Electrophysiology—Transfected cells were transferred to 35-mm dishes containing 140 mM NaCl, 1.7 mM MgCl$_2$, 5 mM KCl, 1.8 mM CaCl$_2$, 25 mM HEPES, pH 7.4, and currents elicited by bath application of agonists at a holding potential of −70 mV were measured in the whole-cell configuration (20) with an Axopatch 200A amplifier (Axon Instruments, Foster City, CA) under computer control using custom programs written in AxoBasic. Patch electrodes contained 145 mM KCl, 2 mM MgCl$_2$, 1 mM EGTA, 25 mM HEPES, pH 7.4, and had resistances of 3–5 megaohms. Agonist and antagonists were dissolved in extracellular solution and delivered to the cell by means of a fast perfusion system (Warner Instruments, Hamden, CT). For experiments involving inhibition of 5-HT-elicited currents by curare, cells were perfused with the desired concentration of curare for 2 min before switching to a solution containing 5-HT and curare.

Dose-response curves from individual cells were normalized to the maximum current and were fit to Equation 4 as described previously (21),

$$\theta = \left[\left(1 + \frac{EC_{50}}{[A]}\right)^n\right]^{-1} \quad \text{(Eq. 4)}$$

where θ is the normalized current at 5-HT concentration [A], EC$_{50}$ is the concentration of 5-HT required to obtain half-maximal current, and n is the apparent Hill coefficient.

RESULTS

Our initial focus was on Trp-89, which is in the homologous position to the two tryptophan residues in the *Torpedo* AChR

GlyR α1	Y	R	V	N	I	F	L	R	Q	Q	W
GABA$_A$R α1	V	Y	T	L	M	Y	F	Q	Q	S	W
AChR α	V	T	T	N	V	R	L	K	Q	Q	W
AChR β	M	S	T	K	V	Y	L	D	L	E	W
AChR γ	L	T	T	N	V	W	I	E	M	Q	W
AChR δ	L	T	T	N	V	W	I	D	H	A	W
5HT$_3$R	L	T	T	Y	I	W	Y	R	Q	Y	W

FIG. 1. **Ligand-gated ion channel sequence alignments of the region surrounding the tryptophans in the acetylcholine receptor photolabeled by curare.** Sequences centered on residues in positions homologous to Trp-55 in the γ and Trp-57 in the δ subunit of the AChR for the indicated receptor subunits are shown. Note that tryptophan is found only in the AChR γ and δ subunits and the 5HT$_3$R. *GlyR,* glycine receptor; *GABA$_A$R*, γ-aminobutyric acid type A receptor.
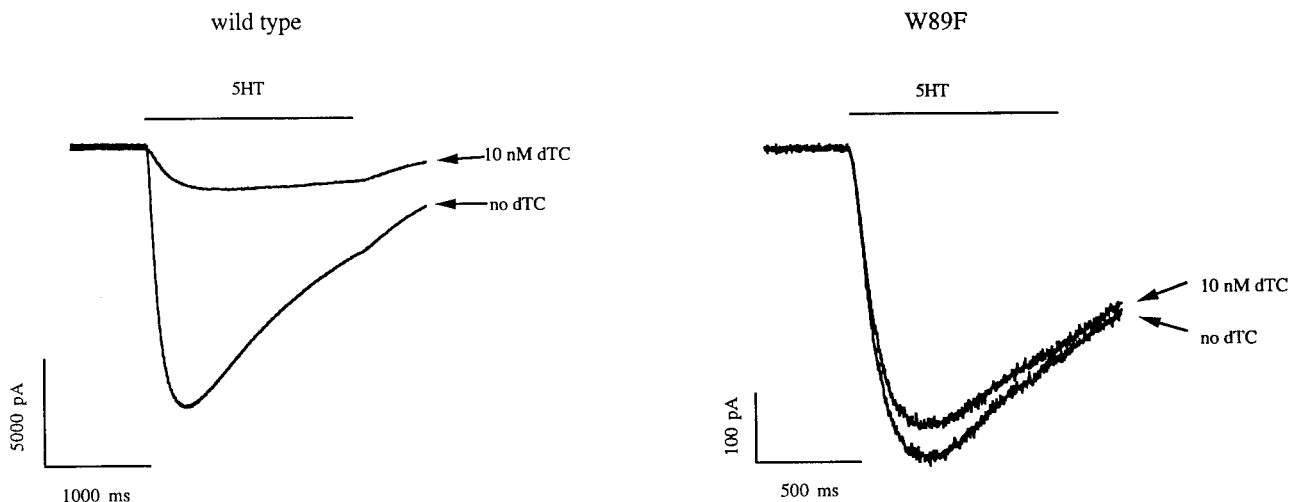
wild type

W89F

FIG. 2. **Amino acid substitution at position Trp-89 markedly reduces curare sensitivity.** Cells expressing either wild type (*left*) or W89F 5HT$_3$Rs were voltage-clamped, and currents elicited by bath application of 1 μM 5HT in the presence or absence of 10 nM curare (*dTC*) were recorded. Note that the mutant receptors are relatively insensitive to curare.

TABLE I
Properties of wild type and W89F 5HT$_3$Rs

Ligand affinities of both wild type and W89F receptors were determined using either saturation isotherms for [^{3}H]granisetron (for granisetron) or competition assays (for curare and serotonin) as described under "Experimental Procedures." Channel activation parameters were determined using the responses to bath-applied serotonin as described under "Experimental Procedures." In both cases, each parameter was obtained using 3–5 determinations at each concentration, and errors represent the error estimates returned by the fitting routine.

	Wild type	W89F
Ligand binding affinities		
Granisetron	1.3 ± 0.1 nM	10.9 ± 2.9 nM
Curare	138 ± 22 nM	1063 ± 179 nM
Serotonin	164 ± 62 nM	215 ± 30 nM
Channel activation		
Serotonin EC$_{50}$	2.4 ± 0.1 μM	2.4 ± 0.1 μM
Hill coefficient	2.2	1.9

that are photolabeled by curare, γTrp-55 and δTrp-57 (10). Initially, Trp-89 was replaced by alanine, which should have little effect on α-helical or β-sheet structure and thus should produce little structural perturbation. However, we were unable to elicit whole cell currents with 5-HT concentrations up to 10 mM from cells transfected with W89A receptors nor were we able to detect specific binding of the antagonist [^{3}H]granisetron in membranes prepared form transfected cells (data not shown). Obviously, substitution of Trp-89 with alanine either interfered with the proper assembly or the functioning of the receptor. We then replaced tryptophan with another aromatic amino acid, phenylalanine, which should represent a less severe substitution. Fig. 2 shows 5-HT-elicited currents recorded from cells transfected with either wild type (Fig. 2, *left*) or W89F (Fig. 2, *right*). Although the activation properties of the two receptors are essentially identical (Table I), the sensitivity to the competitive antagonist curare was markedly different. Wild type receptors were inhibited 85% by 10 nM curare, whereas W89F receptors were inhibited less than 15% under the same conditions.

This reduction in curare sensitivity is also seen in ligand binding studies. Saturation binding isotherms for the competitive antagonist [^{3}H]granisetron were determined for both wild type and W89F receptors (Fig. 3A). The B_{max} value for wild type receptors was on the order of 2–4 pmol/mg of protein, whereas that of the W89F receptors was 1–2 pmol/mg of protein (data not shown). The expression levels of wild type receptors are 5–10 times that reported for N1E-115 neuroblastoma cells (22) or transiently transfected COS-1 (4) cells. The affinity of the W89F receptors for [^{3}H]granisetron was markedly reduced compared with wild type (K_d = 10.9 ± 2.9 nM (W89F) *versus* 1.3 ± 0.1 nM (wild type)).

We examined the effects of the W89F mutation on the affinity of other agonists and antagonists through the use of analysis of the reduction in [^{3}H]granisetron binding in the presence of increasing concentrations of agonists and antagonists. Inhibition of [^{3}H]granisetron binding by curare to wild type and W89F receptors showed a similar reduction in ligand affinity in the mutant (Fig. 3B). The K_i for curare inhibition in wild type receptors was 138 ± 22 nM, whereas the value for W89F receptors was 1063 ± 179 nM, a reduction in affinity comparable with that seen in the [^{3}H]granisetron saturation binding isotherms. A similar reduction in affinity was also observed for another 5HT$_3$R antagonist, MDL-72222 (data not shown). In all three cases, the W89F mutation results in a reduction of ligand affinity. However, when agonists were used in the competition studies, a different picture emerges. No significant difference in affinity between mutant and wild type receptors was observed for the full agonist serotonin (Fig. 3C) or the
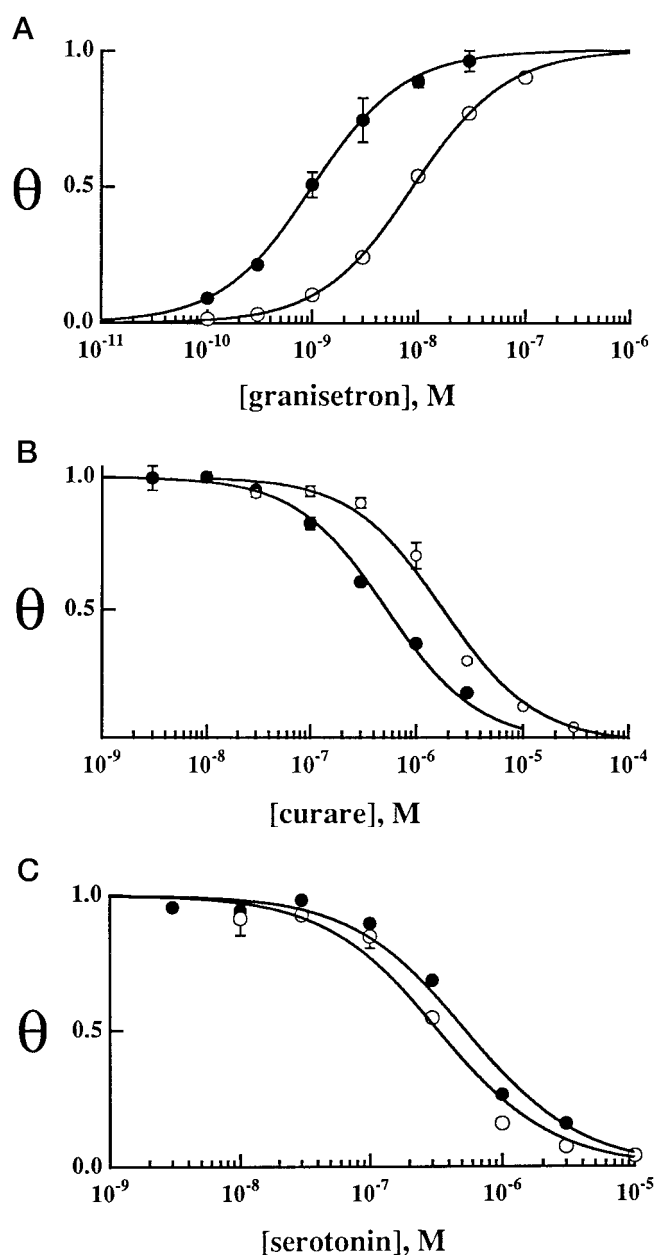

FIG. 3. **Ligand binding properties of wild type and W89F 5HT$_3$Rs.** *A*, saturation isotherms for [^{3}H]granisetron for wild type (●) and W89F (○) receptors were determined as described under "Experimental Procedures." Each *point* represents the mean ± S.E. of 3–5 determinations. The *solid curves* are drawn according to Equation 1 with K_δ values of 1.3 nM (wild type) and 10.9 nM (W89F). *B*, inhibition of [^{3}H]granisetron binding to wild type (●) and W89F (○) receptors by various concentrations of curare are shown. Each *point* represents the mean ± S.E. of 3–5 determinations. The *smooth curves* are drawn according to Equation 2 with IC$_{50}$ values of 535 nM (wild type) and 1800 nM (W895). *C*, inhibition of [^{3}H]granisetron binding to wild type (●) and W89F (○) receptors by various concentrations of serotonin are shown. Each *point* represents the mean ± S.E. of 3–5 determinations. The *smooth curves* are drawn according to Equation 2 with IC$_{50}$ values of 529 nM (wild type) and 329 nM (W895).

partial agonists phenylbiguanide or *m*-chlorophenylbiguanide (data not shown). The serotonin data are consistent with the electrophysiological assays in which no difference in the activation parameters was observed between wild type and W89F receptors.

The above findings suggest that Trp-89 interacts specifically with antagonists but not with agonists. To explore this in more detail, alanine-scanning mutagenesis (15) of this region of the
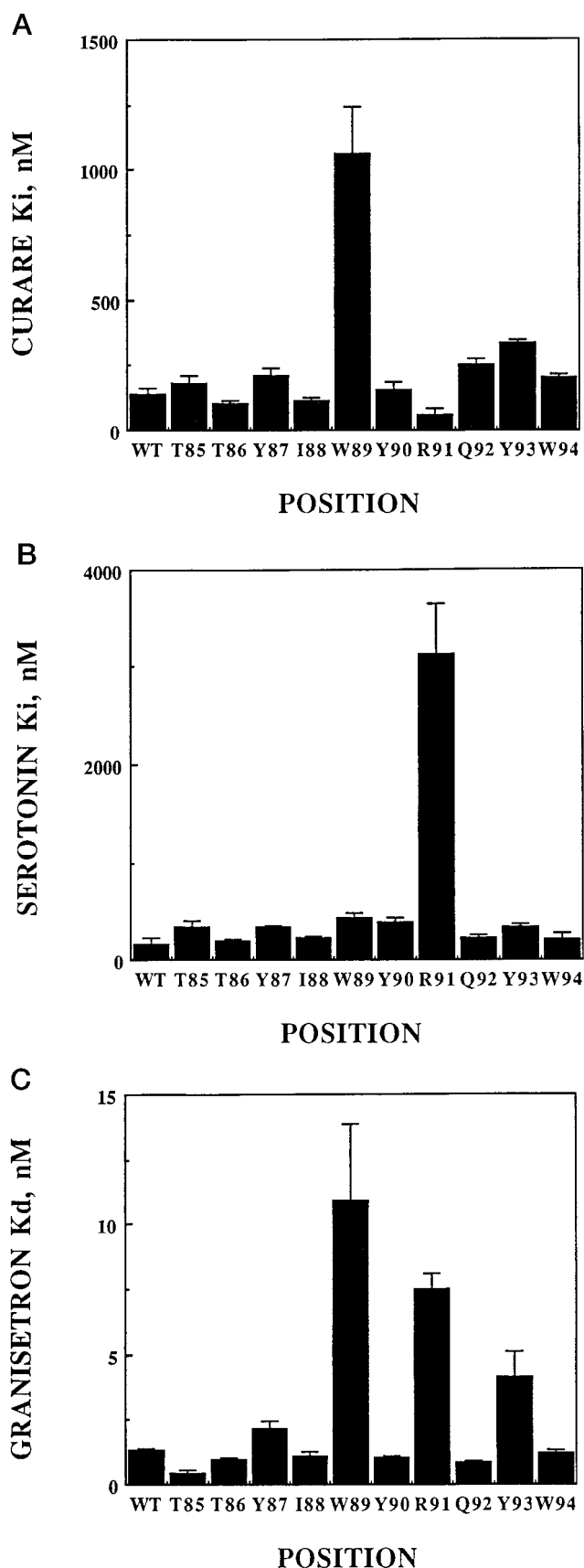
Secondary Structure of 5HT$_3$R Ligand-binding Domain

FIG. 4. **Alanine scan analysis of the region Thr-85 to Trp-94.** Single alanine substitutions were made at the indicated positions (with the exception of Trp-89, which was Phe), and the affinities of curare (*A*), serotonin (*B*), and granisetron (*C*) were determined using 3–5 determinations at each concentration as described under "Experimental Procedures." Each value represents the mean ± the error estimate from the

receptor was carried out. In this approach, functionally important residues are identified by sequential replacement of residues within a specific region with alanine. Alanine is used as the replacement because it eliminates the side chain beyond the β-carbon yet, unlike glycine, generally does not alter main chain conformation. We carried out the analysis on nine residues flanking Trp-89 (Thr-85 to Trp-94) using [^{3}H]granisetron, curare, and serotonin as the ligands to examine the properties of the binding site. All nine alanine-substituted receptors were expressed on the cell surface and formed 5HT$_3$Rs. The receptor density (determined as the B_{max} values for [^{3}H]granisetron binding) of the mutant receptors ranged from 0.1 pmol/mg (Q92A) to 2.2 pmol/mg (T86A).

The results of the alanine scan in terms of ligand affinities are presented in Fig. 4. Each of the three ligands shows a distinct profile. For example, the only substitution that significantly affects the interaction of curare with the receptor is W89F; all other replacements in this region have little effect on affinity. In the case of the agonist serotonin, the only substitution that alters the affinity of the receptor is R91A. Finally, the affinity of [^{3}H]granisetron is reduced by substitutions at both Trp-89 and Arg-91, as well as by a third, Y93A. These results suggest that whereas this region of the receptor is clearly involved in ligand binding, each of these three structurally different ligands interacts in a slightly different fashion with the ligand-binding domain of the receptor. These differences, in turn, can provide some information on some of the structural features of the receptor.

DISCUSSION

Previous studies have suggested the involvement of tryptophan residues in ligand binding in both the AChR (10, 11) and the 5HT$_3$R (14). The data presented in this study demonstrate that Trp-89, which is in the same homologous position as the two tryptophan residues in the AChR that interact with curare (γTrp-55, δTrp-57), plays an important role in curare-5HT$_3$R interactions. The fact that this is a region in the 5HT$_3$R homologous to a domain known to be involved in ligand binding in the AChR lends support to the notion that similar regions in the two receptors are involved in ligand binding.

The data presented in this study suggest that although the region around Trp-89 in the 5HT$_3$R is clearly involved in ligand binding, the specific interactions of various compounds with the binding site may be different. In particular, the fact that in the region Thr-85 to Trp-94 substitution at Trp-89 affects curare, but not serotonin, affinity indicates that there are differential interactions between the binding site and the ligands. This is presumably because of differences in ligand structure and/or positioning within the binding site.

In addition to the notion that the "fine structure" of ligand-receptor interactions may differ for different ligands, our data also provide some insight into the structure of the binding site itself. Unlike curare and serotonin, whose binding is sensitive to mutations at only one residue, the affinity of the antagonist granisetron is affected by mutations at three different residues in this region (Trp-89, Arg-91, Tyr-93). The fact that the relevant positions are in an every-other-residue pattern suggests very strongly that this region of the receptor is in a β-strand conformation. In support of this hypothesis, secondary structure predictions using the Chou-Fasman (17) and Garnier-Robson (18) algorithms predict that this region of the protein should exist in a β-sheet structure. This aspect of the study demonstrates the power of the alanine scan mutagenesis ap-

fitting procedure. Note the different patterns of sensitivity to the mutations.

proach, which can pick up structural features that a more focused single-residue mutagenesis approach might miss.

Tsigelny *et al.* (23) have described a model for the extracellular domain of the nicotinic acetylcholine receptor that is based on sequence similarity to copper-binding proteins of known crystal structure. The region homologous to the domain that we have studied (residues 51–60 in their numbering system) was modeled as an α-helical structure by these authors. However, this region was in one of the four insertions that had to be introduced into the backbone of the copper-binding protein scaffold, and their assignment was based on the fact that in their hands the Chou-Fasman indicates a favorable environment for α-helical structure. In our secondary structure analysis, we did notice that the Chou-Fasman algorithm predicted an α-helical structure for several small stretches in this region of the 5HT₃R but that the helical prediction was weaker than that for a β-strand, which extended through the entire region. Furthermore, an alternate prediction algorithm, the Garnier-Robson method, did not predict any α-helical structure in this region but did predict a β-strand structure. In addition, our own analysis of this particular region of the AChR using the Chou-Fasman and Garnier-Robson algorithms indicated that this particular region of the γ and δ subunits of the AChR should be in the β-strand, not α-helical, configuration. In any case, we feel that our experimental data strongly favor a β-strand structure for this region of the 5HT₃R and, on homology grounds, in all other members of the family, including the AChR.

The data presented here are consistent with the notion that there is a significant amount of structural and functional homology between the AChR and 5HT₃R (and by inference, the other members of the ligand-gated ion channel family). Changeux and co-workers (6) have proposed that the ligand-binding domain of the AChR is formed by several distinct regions of the α and non-α subunits. We envision that the ligand-binding domain of the 5HT₃R is also formed by several different parts of the extracellular domain that are homologous to those regions of the AChR known to be involved in ligand binding. The present study has focused on one of these regions, and the data indicate that this notion that homologous regions are involved in ligand binding is indeed correct. The expansion of our studies to the other regions of 5HT₃R that are homologous to the ligand-binding domains of the AChR will allow us to test the notion that the functional homology of the members of the ligand-gated ion channel family extends to a fairly resolved level. Given the success of our initial foray, as well as our findings concerning the secondary structure of a portion of the binding site, we envision this as a somewhat reciprocating process, in which information obtained from one member of the gene family can be used to guide the studies on another, and vice versa.

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