Aromatic amino acids are important components of the ligand binding site in the Cys loop family of ligand-gated ion channels. To examine the role of tryptophan residues in the ligand binding domain of the 5-hydroxytryptamine$_3$ (5-HT$_3$) receptor, we used site-directed mutagenesis to change each of the eight N-terminal tryptophan residues in the 5-HT$_3$ receptor subunit to tyrosine or serine. The mutants were expressed as homomeric 5-HT$_3$ receptors in HEK293 cells and analyzed with radioligand binding, electrophysiology, and immunocytochemistry. Mutation of Trp$_{90}$, Trp$_{183}$, and Trp$_{195}$ to tyrosine resulted in functional receptors, although with increased EC$_{50}$ values (2–92-fold) to 5-HT$_3$ receptor agonists. Changing these residues to serine either ablated function (Trp$_{90}$ and Trp$_{183}$) or resulted in a further increase in EC$_{50}$ (Trp$_{195}$). Mutation of residue Trp$_{69}$ had no effect on ligand binding or receptor function, whereas mutation of Trp$_{95}$, Trp$_{102}$, Trp$_{121}$, and Trp$_{214}$ ablated ligand binding and receptor function, and all but one of the receptors containing these mutations were not expressed at the plasma membrane. We propose that Trp$_{90}$, Trp$_{183}$, and Trp$_{195}$ are intimately involved in ligand binding, whereas Trp$_{95}$, Trp$_{102}$, Trp$_{121}$, and Trp$_{214}$ have a critical role in receptor structure or assembly.

The 5-HT$_3$ receptor is a member of the Cys loop family of ligand-gated ion channels, which includes nicotinic acetylcholine (nACh), $\gamma$-aminobutyric acid (GABA), and glycine receptors. The predicted 5-HT$_3$ receptor subunit structure shows the expected characteristics of a nACh receptor-type subunit, including a large extracellular N-terminal region, and four putative transmembrane domains (TM1–TM4) with a bulky intracellular loop between TM3 and TM4. 5-HT$_3$ receptor subunits have been cloned from mouse, rat, human, and guinea pig homomeric 5-HT$_3$ receptors were developed using the eukaryotic expression vector pRc/CMV (In Vitrogen, Abingdon, UK) containing the complete coding sequence for the 5-HT$_3$ receptor subunit cloned from NIE-115 cells as described previously (27). Mutagenesis reactions were performed using the Kunkel method (28) and confirmed by DNA sequencing.

**EXPERIMENTAL PROCEDURES**

**Coll Culture and Transient/Stable DNA Transfection—**Human embryonic kidney (HEK293) cells were cultured on 90-mm tissue culture plates in Dulbecco’s modified Eagles medium/F-12 medium (1:1) (Life Technologies, Inc.) supplemented with 10% fetal calf serum (Sigma) at 37°C and 7% CO$_2$ in a humidified atmosphere. HEK293 cells stably expressing 5-HT$_3$ receptors were developed using the eukaryotic expression vector pRcCMV (In Vitrogen, Abingdon, UK) containing the complete coding sequence for the 5-HT$_3$ subunit from NIE-115 cells as described previously (27). Mutagenesis reactions were performed using the Kunkel method (28) and confirmed by DNA sequencing. For transient transfections, HEK cells at 60–70% confluency (~48 h post passage) were transfected with WT or mutant plasmid DNA by electroporation experiments. For radioligand binding studies cells were grown on 90-mm plates (Falcon) and harvested 3 days after transfection. For electrophysiology experiments cells were grown on 35-mm plates, and recordings were performed 1–4 days post-transfection. In immunofluorescence experiments cells were grown on 22-mm diameter glass coverslips in 35-mm plates and stained 3 days post-transfection.

**Radioligand Binding—**Transfected HEK293 cells were washed twice with phosphate-buffered saline at room temperature; all subsequent steps were carried out at 1–4°C. Cells were scraped into 1 ml of HEPES buffer (10 mM, pH 7.4) containing the following proteinase inhibitors: 1 mM EDTA, 50 $\mu$g/ml soybean trypsin inhibitor, 50 $\mu$g/ml bacitracin, and 0.1 mM phenylmethylsulfonyl fluoride. Harvested cells were washed in HEPES with protease inhibitors and frozen at −20°C. After thawing,
they were washed twice with HEPS buffer and resuspended, and 50 μg of cell membranes were incubated in 0.5 ml of HEPS buffer containing [3H]granisetron (81 Ci/mmol, DuPont) ranging in concentration from 0.05 to 40 nM, or [3H]mCPBG (26 Ci/mmol) and mCPBG (26 Ci/mmol) were from DuPont. All other chemicals were obtained from Sigma. 5-HT hydrochloride was from Research Bio- Technologies, Inc. (Paisley, UK) except fetal calf serum, which was from Sigma (Poole, UK). mCPBG and 2-methyl-5-HT (2-Me-5-HT) were from Tocris Chemicals (Bristol, UK). [3H]Granisetron (81 Ci/mmol, DuPont) and mCPBG (26 Ci/mmol) were from DuPont. All other reagents were obtained from Sigma.

Electrophysiological Procedures—Patch electrodes were made from filamented glass capillary tubing (Clark Elektronik, Darmstadt, Germany) controlled by Pulse software (HEKA). Tryptophan residues in the 5-HT3R ligand binding domain

| Receptor | [3H]Granisetron | [3H]mCPBG |
|----------|-----------------|-----------|
| Wild type | 0.17 ± 0.03 | 1.03 ± 0.01 | 1.48 ± 0.09 | 1.07 ± 0.02 |
| W60S | 0.21 ± 0.05 | 0.84 ± 0.15 | 1.31 ± 0.13 | 0.90 ± 0.06 |
| W90Y | 0.92 ± 0.06 | 0.92 ± 0.03 | 8.24 ± 0.73 | 1.00 ± 0.003 |
| W90S | ND | ND | ND | ND |
| W183Y | ND | ND | ND | ND |
| W183S | ND | ND | ND | ND |
| W195Y | 8.18 ± 2.4 | 1.24 ± 0.18 | 2.66 ± 0.62 | 0.84 ± 0.22 |
| W195S | 1.25 ± 0.07 | 1.37 ± 0.14 | 2.47 ± 0.28 | 0.72 ± 0.17 |

* Significantly different from WT (p ≤ 0.05).
ND, not detected.

TABLE I
Effect of tryptophan mutations on 5-HT3 receptor radioligand binding

Values are the means ± S.E. (n ≥ 3).

Wild Type and Try60 Mutant Receptors—Radioligand binding studies using membranes from WT-transfected HEK293 cells revealed that [3H]granisetron and [3H]mCPBG label a homogeneous population of binding sites with high affinity: KD = 0.17 ± 0.03 nM and 1.48 ± 0.09 nM respectively (n = 3). Hill coefficients were not significantly different to unity. There was no difference in radioligand binding parameters for either [3H]granisetron or [3H]mCPBG because of modification of the 5-HT3 receptor W60 to serine (Table I). Whole cell patch clamp revealed that 5-HT and the 5-HT3 receptor agonist mCPBG elicited rapid transient inward currents from cells transfected with WT DNA (Fig. 2). From concentration-effect curves EC50s were 2.10 ± 0.40 μM and 0.81 ± 0.09 μM for 5-HT and mCPBG respectively. The EC50 and nH values for W60S 5-HT3 receptor constructs were not significantly different to WT (Table II).

Effect of Mutations at Positions 90, 183, and 195—Using membranes prepared from W90Y-transfected HEK293 cells, a 5.4-fold increase in KD for [3H]granisetron was observed compared with WT; the Hill coefficient was not significantly changed. The change in antagonist affinity was reflected by a similar change in agonist affinity: KD for [3H]mCPBG increased 5.6-fold, with no effect on the Hill coefficient. Binding of both radioligands was ablated by the mutations W90S, W183Y, and W183S. W195Y and W195S mutations did not affect the [3H]mCPBG binding affinity but significantly increased the KD for [3H]granisetron binding; these latter increases were not significantly different between the two mutants (Table I).

The EC50 values for the W90Y mutant, stably transfected into HEK293 cells, were increased 3.8- and 2.5-fold as compared with WT for 5-HT and mCPBG, respectively (Fig. 3), whereas the EC50 for the partial agonist 2-Me-5-HT was not altered; EC50 values were 12.7 ± 0.45 μM and 12.9 ± 1.98 μM for WT and W90Y, respectively (n = 3). The Hill coefficients for all three agonists were not significantly changed by the W90Y mutation. The mutant W90S, when expressed in HEK293 cells, did not show any response to 5-HT3 (±1 μM) or mCPBG (±500 μM). The electrophysiological properties of W183Y, W183S, W195Y, and W195S mutations were also examined in transfected HEK293 cells. For the W183Y mutation, EC50 values for 5-HT and mCPBG were increased 92- and 24-fold, respectively, compared with WT, whereas there was no response to application of either agonist for the W183S mutation. The W195Y mutation resulted in 3.9- and 2.1-fold increases in EC50 for 5-HT and mCPBG, respectively, whereas the W195S mutation caused 9.0- and 2.7-fold increases in EC50, respectively. The Hill coefficients were not affected by W183Y, W195Y, or W195S mutations for either agonist (Table II). The time course of desensitization for these mutations was slower than WT (Fig. 2), suggesting that the mutations had resulted in changes to the

\[
\text{KD} = \frac{[L]}{[L]_t} \times K_n = \frac{[L]}{[L]_t} + K_n \text{homogeneous population of binding sites with high affinity;}\]

\[
B = B_{max} \times \frac{[L]}{[L] + K_n} \text{maximum concentration of radioligand, and } n \text{ is the Hill coefficient.}\]

\[
I = I_{max} \times \frac{[L]}{[L] + K_n} \text{current at conformational change, [L] is the free concentration of radioligand, and } n \text{ is the Hill coefficient.}\]

\[
\text{Hill coefficient was not significantly changed. The change in antagonist affinity was reflected by a similar change in agonist affinity: KD for [3H]mCPBG increased 5.6-fold, with no effect on the Hill coefficient. Binding of both radioligands was ablated by the mutations W90S, W183Y, and W183S. W195Y and W195S mutations did not affect the [3H]mCPBG binding affinity but significantly increased the KD for [3H]granisetron binding; these latter increases were not significantly different between the two mutants (Table I).}\]

\[
\text{Effect of tryptophan mutations on 5-HT3 receptor radioligand binding}\]

Values are the means ± S.E. (n ≥ 3).

| Receptor | [3H]Granisetron | [3H]mCPBG |
|----------|-----------------|-----------|
| Wild type | 0.17 ± 0.03 | 1.03 ± 0.01 | 1.48 ± 0.09 | 1.07 ± 0.02 |
| W60S | 0.21 ± 0.05 | 0.84 ± 0.15 | 1.31 ± 0.13 | 0.90 ± 0.06 |
| W90Y | 0.92 ± 0.06 | 0.92 ± 0.03 | 8.24 ± 0.73 | 1.00 ± 0.003 |
| W90S | ND | ND | ND | ND |
| W183Y | ND | ND | ND | ND |
| W183S | ND | ND | ND | ND |
| W195Y | 8.18 ± 2.4 | 1.24 ± 0.18 | 2.66 ± 0.62 | 0.84 ± 0.22 |
| W195S | 1.25 ± 0.07 | 1.37 ± 0.14 | 2.47 ± 0.28 | 0.72 ± 0.17 |
rate constants between the open and desensitized states of the receptor, perhaps indicating decreased stability of the desensitized state in the mutant receptors. Changes in stability of the different states of the receptor are likely to affect the equilibrium binding data, which depends on the interplay between these different states at equilibrium; this interplay will differ in the presence of agonists, where desensitization is obligatory, and antagonists, which may bind preferentially to either the closed or desensitized state. Thus, if our hypothesis is correct, the change in affinity that we observed for antagonist but not agonist binding in the Trp195 mutants suggests that in these receptors granisetron, an antagonist, may have a stronger preference than mCPBG for the desensitized state of the receptor.

### Table II

| Receptor       | 5-HT<sub>3</sub> | mCPBG |
|----------------|----------------|-------|
|                | EC<sub>50</sub> | n<sub>H</sub> | EC<sub>50</sub> | n<sub>H</sub> |
| Wild type      | 2.10 ± 0.40    | 2.22 ± 0.20 | 0.81 ± 0.09 | 1.97 ± 0.20 |
| W60S           | 2.26 ± 0.21    | 1.71 ± 0.14 | ND           | ND           |
| W90Y           | 8.02 ± 0.30<sup>a</sup> | 2.02 ± 0.11 | 2.00 ± 0.10<sup>a</sup> | 2.14 ± 0.13 |
| W90S           | ND<sup>a</sup> | ND        | ND           | ND           |
| W183Y          | 194 ± 19.2<sup>a</sup> | 1.92 ± 0.26 | 19.6 ± 2.8<sup>a</sup> | 2.05 ± 0.18 |
| W183S          | ND           | ND        | ND           | ND           |
| W195Y          | 8.09 ± 0.93<sup>a</sup> | 2.74 ± 0.25 | 1.68 ± 0.38<sup>a</sup> | 1.58 ± 0.20 |
| W195S          | 18.8 ± 2.45    | 2.58 ± 0.15 | 2.18 ± 0.16<sup>a</sup> | 2.19 ± 0.27 |

<sup>a</sup> Significantly different from WT (p < 0.05).
<sup>b</sup> ND, not detected.

FIG. 1. Amino acid sequence of the N-terminal (ligand binding) domain of the 5-HT<sub>3</sub> receptor. Sequence is shown to transmembrane domain 1, with tryptophan residues highlighted in bold type. The putative signal sequence is shown underlined. Inset represents the putative transmembrane topology of the 5-HT<sub>3</sub> receptor, illustrating extracellular N and C termini and transmembrane domains 1–4.

FIG. 2. Electrophysiological responses of 5-HT<sub>3</sub> receptor mutants W183Y and W195S compared with WT. Responses of single cells (representative of at least four different cells) are shown at maximal and EC<sub>50</sub> concentrations of 5-HT.

FIG. 3. Dose-response curves for 5-HT<sub>3</sub> WT (filled circles) and mutant W60S (open circles), W90Y (crosses), W183Y (filled squares), W195S (open squares), and W183Y (filled triangles) receptors generated using the agonists 5-HT (A) and mCPBG (B). Responses were measured at −60 mV holding potential. Each data point is the mean of responses from ≥4 cells, and error bars indicate S.E. Curves were fitted with the Hill equation and EC<sub>50</sub> values, and Hill coefficients are given in Table II.

Tryptophan Residues in the 5-HT<sub>3</sub>R Ligand Binding Domain

The values are the means ± S.E. (n ≥ 4).
receptors showed no specific binding with either ligand. Furthermore, none of the mutants W95Y, W95S, W102Y, W102S, W121Y, W121S, W214Y, and W214S, when transiently transfected into HEK293 cells, showed electrophysiological responses to ≤1 mM 5-HT.

Immunolocalization of WT and Mutant 5-HT₃A Receptors—Receptors that give rise to agonist-induced currents in transfected HEK293 cells are clearly expressed at the plasma membrane. To determine whether mutants that did not respond to agonist or demonstrate detectable radioligand binding are correctly oligomerized and expressed at the plasma membrane, the localization of nonfunctional mutant subunits was studied using a 5-HT₃A receptor N-terminal specific antibody, pAb120 (30). Cytoplasmic 5-HT₃ receptor-specific staining was observed in cells with permeabilized membranes expressing WT and all mutant constructs tested (Figs. 4 and 5). pAb120 immunolabeled cells, transfected with WT, functional mutants (data not shown) and nonfunctional mutants W90S, W183S, and W214Y with nonpermeabilized membranes, acquired a characteristic narrow ring of pAb120 labeling around the membrane. This strong fluorescent signal from the membrane was not observed in cells that did not express receptor or cells that expressed any of the other nonfunctional mutant constructs. The fluorescence in the membrane was granular in appearance, possibly corresponding to aggregations of receptors in the membrane.

DISCUSSION

To identify the functional roles of tryptophan residues in the N-terminal domain of the 5-HT₃A receptor, we substituted the eight tryptophans in this domain (Fig. 1) to tyrosine and/or serine. The pharmacology, electrophysiology, and localization of the altered receptors were examined using the HEK293 cell expression system. The data we have presented suggest that three of the tryptophan residues, Trp90, Trp183, and Trp195, play a role in ligand binding, whereas the four residues Trp95, Trp102, Trp121, and Trp214 are critical for the correct receptor assembly and/or structure.

The nonconservative substitution of Trp60 for serine results in mutant receptors that have radioligand binding and electrophysiological properties not discernible from the WT receptor, suggesting that Trp60 does not play a role in ligand binding and appears to have little or no structural significance. Alignments of the 5-HT₃, nACh, GABAₐ, and glycine receptors (Fig. 6) show that Trp60 of the 5-HT₃A receptor subunit does not align with tryptophanyl, aromatic, or ligand binding residues from any other members of the nACh-type LGIC family. These findings suggest that Trp60 is not an important residue for either the structure or the function of the 5-HT₃ receptor.

Mutations at Positions 95, 102, and 121—The effects of mutation at these positions to tyrosine and serine were indistinguishable from each other. All six substitutions resulted in receptors that did not bind [³H]granisetron or [³H]mCPBG, did not respond to 5-HT, and were not expressed at the plasma membrane, although high levels of intracellular protein expression were observed for all of the mutant receptors (Fig. 5).
These observations are consistent with subunits not assembling or assembling incorrectly or fully assembled pentamers not being sorted to the plasma membrane. The importance of hydrophobic amino acids in the N-terminal domain for mediating subunit interactions in receptor assembly has been demonstrated in the glycine (32) and nACh (33) receptors, and it is likely that in the 5-HT3 receptor, Trp 90, and Trp 121 are strictly conserved in all binding and nonbinding nACh, GABA A, and glycinergic subunits, whereas Trp 102 residue is conserved in most nACh subunits and is otherwise represented as an aromatic residue in the other subunits of this receptor family (Fig. 6). These canonical residues may therefore create an N-terminal domain backbone structure that is common to all of the LGICs in this family (17, 19, 34, 35).

Mutations at Position 214—The mutation W214Y resulted in a receptor which was expressed at the plasma membrane but did not bind radiolabeled granisetron or mCPBG and did not respond to 5-HT in electrophysiological assays. The effect of the W214Y mutation was more severe, resulting in no binding, function, or expression at the plasma membrane. These data suggest that an aromatic residue is necessary at position 214 for correct receptor expression but is not sufficient to retain function. A tryptophan residue at an equivalent position to Trp 214 is conserved in all binding and nonbinding subunits of the nACh receptor (Fig. 6), although aromatic amino acids are not present at the homologous position in either of the anionic receptors. This tryptophan residue may therefore be an important determinant of the tertiary structure of the N-terminal domain of cationic, but not anionic, channels.

Mutations at Position 90—The W90Y mutation resulted in a receptor with decreased radioligand binding affinities and agonist responses, whereas the replacement of tryptophan with serine caused radioligand binding and agonist responses to be abolished without affecting receptor expression at the plasma membrane. In the W90Y mutant, the similar increase in mCPBG EC50 (agonist binding receptor in the resting, activated state) and Kp (agonist binding receptor in the desensitized state) suggests that the effects of this mutation may be due to a binding site modification rather than an effect on the mechanisms of channel gating. Furthermore, Hill coefficients for radioligand binding and electrophysiological experiments were not altered by the W90Y mutation. This hypothesis is supported by the observation that the W90Y mutation did not change the EC50 for the partial 5-HT3 receptor agonist 2-Me-5-HT, and these data also suggest that 2-Me-5-HT binds to the receptor via interactions different from those of the full agonists, 5-HT and mCPBG. The observation that W90S mutant receptors reach the plasma membrane but do not bind ligands suggests that an aromatic group at this position is essential for ligand binding, either because of its interaction with the ligand or for the formation of the correct structure of the ligand binding pocket.

All 5-HT3 receptor ligands possess an aromatic ring containing a polar group, and a basic nitrogen atom (36). Our data are consistent with a direct interaction of 5-HT3 receptor ligands with Trp 90, and we suggest this is via cation π interactions between the aromatic tryptophan and the basic nitrogen present in 5-HT3 ligands, similar to the ligand binding mechanisms postulated for the binding of the quaternary ammonium ion of ACh to aromatic residues of the nAChR (37, 38). This hypothesis explains the reduction in affinity for ligands caused by the W90Y mutation, because the indole of tryptophan provides a larger and more intense region of negative electrostatic potential than a simple benzene ring, making tryptophan a more attractive cation binding site than tyrosine. The ablation of ligand binding by replacement of Trp 90 with serine is consistent with this hypothesis, because it would result in the complete removal of this putative cation binding site.

Other possible ligand stabilization mechanisms that could explain our data include dispersion, i.e. a mutual synchronization of fluctuating charge in the overlapping indole rings, or a dipole-induced dipole interaction between the polar group of the ligand and the indole of the tryptophan. Both of these interactions would be weaker than the cation π bond that we propose and are therefore probably less likely, although further experimentation and/or determination of the three-dimensional structure of the binding site is required to confirm or disprove our hypothesis.

A recent study also supports a role for Trp 90 in ligand binding and further uses alanine scanning mutagenesis to provide evidence that this region may be in a β-strand conformation (39). Trp 90 aligns with the nAChR α7 Trp 54, which is proposed to form a complimentary component of the ligand binding site, i.e. contributed by an adjacent subunit (24). α7 Trp 54 is highly conserved in nACh subunits that are expected to contribute a complimentary ligand binding loop, although it is also conserved in many of the neuronal α subunits (Fig. 6). An aromatic residue, phenylalanine, occupies this position in GABA and glycine receptor subunits, and in the former has been shown to contribute to the binding site for GABA (40, 41). Thus the data presented for the 5-HT3α Trp 90 residue extend the identified structural and functional homologies between the receptors of this LGIC superfamily in addition to their considerable sequence homologies.

Mutation at Position 183—Mutation of Trp 183 to tyrosine caused a large increase in agonist EC50 values and abolished radioligand binding, whereas the W183S mutant had no apparent ligand binding or function but was expressed at the plasma membrane. The cooperative nature of ligand binding was unaffected by the W183Y mutation, suggesting that its effects are unlikely to be manifest via a structural change in the receptor. Thus residue Trp 183 appears to be involved in ligand binding, probably via cation π interactions as postulated above for the Trp 90 residue.

The tryptophan at position 183 of the 5-HT3 receptor sub-
unit aligns with tryptophan residues in nACh α and β (except muscle type) subunits but is not conserved in the 5-HT$_3$A receptor subunit nor in the nonbinding nACh subunits γ, δ, and ε. Trp$^{183}$ of the 5-HT$_3$A receptor subunit aligns with phenylalanine residues in the glycine receptor and tyrosines in the GABA$_A$ receptor (Fig. 6). These latter aromatic residues have been implicated in ligand binding for the nACh (14, 21), GABA$_A$ (42), and glycine receptors (43). Thus 5-HT$_3$A receptor subunit Trp$^{183}$ and its homologues are the first residues that have been demonstrated to be important for ligand receptor recognition in both the cationic and anionic receptors of this LGIC family. Because the largest change in EC$_{50}$ of the functional tryptophan mutants examined in this study was caused by the W183Y mutation, and the removal of the aromatic residue at this position ablated ligand binding, we propose that this residue is the most important tryptophan in 5-HT$_3$ receptor ligand binding site. Recently the equivalent tryptophan in this residue is the most important tryptophan in 5-HT$_3$ receptor subunit Trp183 and its homologues are the first residues that pose that changing Trp195 specifically effects a region of the binding pocket that has some importance for ligand binding, Trp90, Trp183, and Trp214. The findings of this study further exemplify the high degree of structural and functional homology between the receptors in the Cys loop LGIC family and provide insights toward the subtle differences that may be responsible for the characteristic 5-HT$_3$ receptor ligand binding profile.

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