Prediction of 5-HT_3 Receptor Agonist-Binding Residues Using Homology Modeling

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ABSTRACT 5-HT_3 receptors demonstrate significant structural and functional homology to other members of the Cys-loop ligand-gated ion channel superfamily. The extracellular domains of these receptors share similar sequence homology (~20%) with *Limnaea* acetylcholine binding protein, for which an x-ray crystal structure is available. We used this structure as a template for computer-based homology modeling of the 5-HT_3 receptor extracellular domain. AutoDock software was used to dock 5-HT into the putative 5-HT_3 receptor ligand-binding site, resulting in seven alternative energetically favorable models. Residues located no more than 5 Å from the docked 5-HT were identified for each model; of these, 12 were found to be common to all seven models with five others present in only certain models. Some docking models reflected the cation-π interaction previously demonstrated for W183, and data from these and other studies were used to define our preferred models.

INTRODUCTION

The 5-HT_3 receptor is a member of the Cys-loop family of LGIC. This family of proteins, which includes nACh, $GABA_A$, and glycine receptors, are responsible for fast synaptic transmission at chemical synapses and are the target sites of many neuroactive drugs; however, we do not yet know the molecular details of their structure. The receptors are pentamers, and are usually constituted of 2–4 different subunits; each subunit has a large extracellular N-terminal region and four putative transmembrane domains (M1–M4). Two 5-HT_3 receptor subunits, A and B, have been characterized (see Reeves and Lummis, 2002, for review), and receptors appear to be able to function as either homo-oligomeric (A only) or hetero-oligomeric (A and B) subunit complexes (Davies et al., 1999). 5-HT_3 receptors have been proposed to be evolutionarily the oldest members of the Cys-loop LGIC family, and this, combined with the ability of these receptors to function as homo-oligomers, has meant that 5-HT_3 receptors provide a useful model system for understanding critical features of all Cys-loop receptors (Reeves and Lummis, 2002). Most work to date on this family of proteins has been performed using nACh receptors, yet despite many years of study, structural details of the protein-ligand interactions at the molecular level remained unknown. Evidence from early biochemical and mutagenesis studies on nACh receptors indicated that it was the extracellular domain that contained the ligand-binding site, a hypothesis that was later confirmed by the construction of a chimaeric protein consisting of the N-terminal domain of the α7 neuronal nACh receptor subunit linked to the C-terminal portion of the 5-HT_{3A} receptor subunit: the resulting chimera possessed nACh receptor pharmacological properties and 5-HT_3 receptor channel properties (Eiselé et al., 1993). The determination of the structure of the AChBP (Brejc et al., 2001), which is homologous not only to the extracellular domain of the nACh receptor but also to those of other LGIC including the 5-HT_3 receptor, allows definition of the ligand-binding domain at a greater level of detail than before.

Computer-aided modeling of the extracellular domain of 5-HT_3 receptor has been attempted previously (Menziani et al., 2001; Gready et al., 1997), but the structure of AChBP suggests that these models may not reflect the true binding site. Several homology-based models of the nACh receptor and $GABA_A$ receptor have been recently constructed (Le Novère et al., 2002; Cromer et al., 2002; Schapira et al., 2002). In this paper, we present a model of the 5-HT_3 receptor extracellular domain constructed by homology with AChBP, and discuss the accuracy of the alternative docking models for 5-HT in relation to experimental evidence and sequence alignment data.

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Abbreviations used: 5-HT_3, 5-hydroxytryptamine$_3$; LGIC, ligand-gated ion channel; ACh, acetylcholine; AChBP, acetylcholine binding protein; nAChR, nicotinic acetylcholine receptor; GABA, gamma-amino butyric acid; ECD, extracellular domain.

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MATERIALS AND METHODS

Sequence alignment

A sequence alignment of the extracellular domain of the 5-HT_3 receptor and AChBP was generated using the program FUGUE (Shi et al., 2001), which utilizes environment-specific substitution tables and structure-dependent gap penalties.

Modeling

The three-dimensional model of the extracellular region of the 5-HT_3 receptor was built using MODELLER (Sali and Blundell, 1993) based on

the crystal structure of AChBP determined to 2.7 Å. The pentamer was generated by superimposing the model onto each protomer of AChBP; special care was taken not to alter the coordinate axes of reference. The generated pentamer model was then energy minimized in SYBYL using the AMBER force field (Weiner et al., 1984) by moving side chains alone, to relieve short contacts at the interprotomer interfaces. Electrostatic terms were also included in these minimization cycles.

Ligand docking

The structure of 5-HT was constructed with idealized geometry using MacroModel (Mohamadi et al., 1990), with hydrogen atoms added assuming a pH of 7. The resultant structure, together with the modeled 5-HT$_3$R, were used as input for AutoDock 3.05 (Goodsell and Olson, 1990; Morris et al., 1998). The number of points used in each Cartesian direction was 60, thus giving a spacing of 0.375 Å and a total of 216,000 points. The grid center was manually set to an arbitrary point within the binding site space. Ten genetic algorithm runs were performed on each docking exercise, with a population size of 50, and the maximum number of generations was set to 27,000. Seven docked structures were obtained. These structures were then used as input for a program written by one of us (P.-L.C.) to extract all amino acids that possess at least one atom within 5 Å of the 5-HT ligand. Potential hydrogen-bonding interactions were identified using SwissPDB-Viewer (Guex and Peitsch, 1997). Hydrogen bonds between 1.20 and 2.76 Å were allowed with a minimum angle of 120°. Figures were rendered using POV-Ray 3.1 for Macintosh.

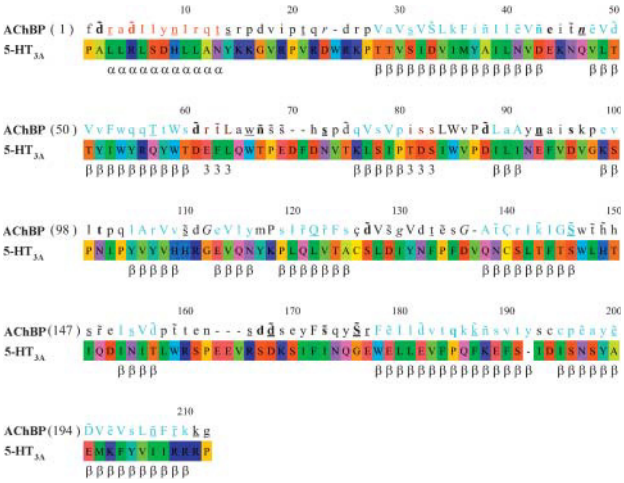


FIGURE 1 Alignment of the amino acid sequence of the extracellular domain of the 5-HT$_{3A}$ receptor subunit with that of AChBP. The alignment is annotated using the program JOY (Mizuguchi et al., 1998). Key to alignment: α-helix, red x; β-strand, blue x; 3_{10} helix, maroon X; solvent accessible, lower case X; solvent inaccessible, upper case X; hydrogen bond to main-chain amide, boldface X; hydrogen bond to main-chain carbonyl, underlined X; disulfide bond, cedilla ç; positive ϕ-torsion angle, italic X.

RESULTS

The sequence identity between the 5-HT$_3$ receptor ECD and AChBP, as shown in Fig. 1, is relatively low (19% for the α-subunit), although this value is similar to that between AChBP and ACh receptor subunit ECDs (an average of 22%). However, the predicted secondary structure of a typical nACh subunit ECD has 61.2% identity with AChBP, and it has been calculated that the secondary structure similarity between AChBP and the α7 nACh receptor subunits is around 80% (Le Novère et al., 2002). Thus the secondary structure of the closely related 5-HT$_3$ receptor ECD, and indeed all Cys-loop LGIC ECDs, is likely to be well represented by the AChBP structure. Our model of the 5-HT$_3$ receptor extracellular domain therefore adopts an immunoglobulin-like fold similar to that found for AChBP (Grutter and Changeux, 2001).

The actual location of the agonist-binding site in AChBP has not been finally determined, as the structure has not yet been crystallized with ACh bound. However, a HEPES molecule was identified in the putative binding pocket. As this molecule has a charged amine like ACh, and the residues associated with the binding site were those previously described from labeling and mutagenesis studies, it seems highly likely that this will prove to be the binding pocket of the protein. This region, which is located at the subunit interfaces, is indicated in Fig. 2 *A*. The relative orientations of loops A–E, all of which contribute to the binding site in our models, are shown in Fig. 2 *B*.

The model of the binding site of 5-HT can be described as a cleft with a predominance of aromatic residues. Of these, F226 and W90 are toward the membrane or lower side of the cleft, whereas W183, Y234, and Y143 are toward the top of the cleft and therefore would be closer to the presynaptic cell. The docking procedure resulted in seven energetically favorable models for positioning 5-HT in this binding site. In models 1–3 (Fig. 3), 5-HT is oriented such that the primary amine is in the lower part of the cleft, with the rings of 5-HT sandwiched between the aromatic rings of Y234 and W183, and there is the potential to form a hydrogen bond with the main chain carboxyl of S182. The hydroxyl of 5-HT appears to be in a hydrophilic pocket formed by R92, D229, and Q151. The models differ in having subtle variations in the locations of the primary amine and the rings in the cleft, and this is reflected in the different lengths of the potential hydrogen bond with S182: for model 1, 2.4 Å; for model 2, 2.6 Å; and for model 3, 2.2 Å.

In models 4 and 5 (Fig. 3), 5-HT is oriented such that the rings are at the lower part of the cleft and the primary amine is between W183 and Y234. The hydroxyl group is located in a hydrophilic pocket formed by T179, E236, and N128, with the potential to form hydrogen bonds with the latter two. There is also the potential to form a third hydrogen bond with the main chain carboxyl of S182 (of 2.0 Å, model 4) or W183 (of 2.3 Å, model 5). Models 4 and 5 differ by subtle changes in the location of the primary amine—in model 4, the nitrogen is 4.1 Å from the nearest carbon of the Y234 ring and 4.8 Å from the nearest carbon of the W183 benzene ring, whereas in model 5 the distances are 3.9 Å and 5.2 Å, respectively. There is also a difference in the twist of the rings, which would result in different length hydrogen bonds

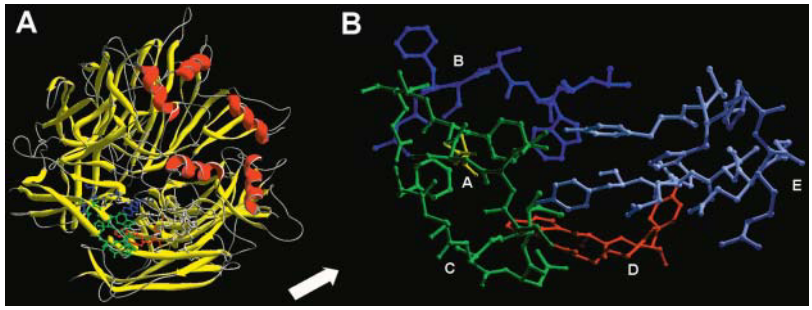


FIGURE 2 Homology model of the 5-HT_3 receptor extracellular domain. (*A*) Model of 5-HT_3 receptor based on AChBP showing the location of the putative ligand-binding pocket. The white arrow points perpendicular to the membrane in the plane of the ion channel away from the membrane. (*B*) The five ligand-binding loops A–E for 5-HT_3 receptor as defined for the nACh receptor. Key: loop A (*yellow*); loop B (*blue*); loop C (*green*); loop D (*red*); loop E (*gray*).

with the keto group of N128 (2.8 Å and 2.5 Å for models 4 and 5, respectively).

In model 6, the indole N atom of 5-HT is toward the top of the cleft, and is similarly located in model 7, although here 5-HT has rotated ~180°. The primary amines are therefore at quite distinct locations at either upper right of the cleft with the N atom 3.2 Å from the nearest atom of Y143 (model 6), or to the lower left at a distance of 3.7 Å from the nearest atom of F226 (model 7). The hydroxyl groups, however, are both located close to Y153: in model 6 there is the potential to form a hydrogen bond of length 2.3 Å, whereas in model 7 it is 3.3 Å from the nearest carbon atom. The former is the only potential hydrogen bond we identified in model 6, and in model 7 there is also only one potential hydrogen bond, between the indole N atom and the backbone carbonyl of S182.

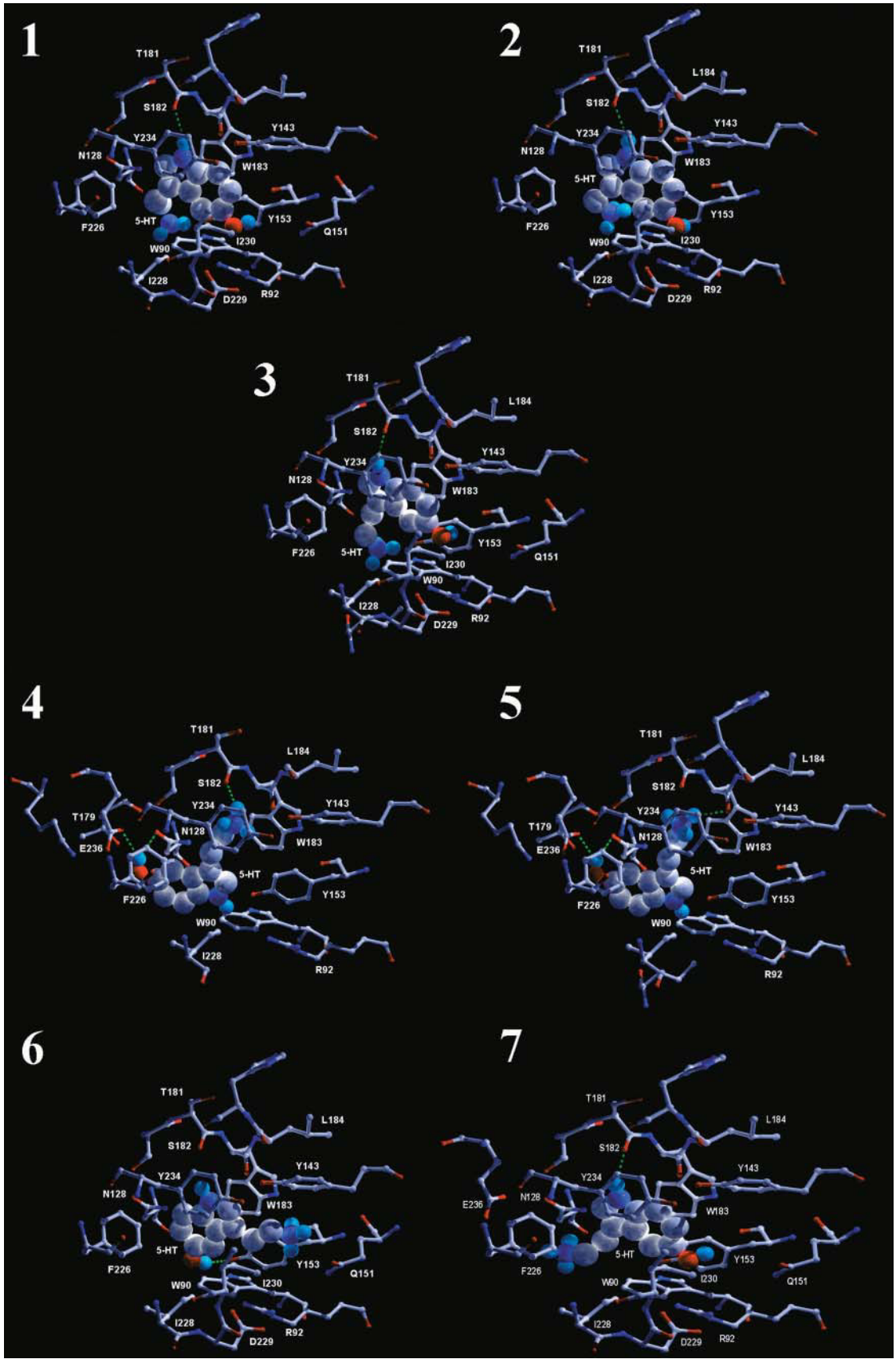
DISCUSSION

We have used information available from the x-ray crystal structure of AChBP to model the extracellular domain of the 5-HT_3 receptor. This region contains the binding sites for 5-HT, which lie at the subunit interfaces. Simulated docking of 5-HT with our model of the binding site using the program AutoDock revealed seven energetically favorable positions of 5-HT. Examination of the residues within 5 Å of 5-HT showed that many (12 out of 17) were common to all positions (see Table 1), and most of these residues, as expected, fall in the binding loop regions that have been proposed to constitute the binding site (Fig. 2 *B*). Our data also identify residues that have the potential to form hydrogen bonds with the agonist, and therefore may be involved not only in its correct localization but also in transducing agonist binding into channel opening. The results show that homology modeling can be valuable in locating residues involved in agonist binding, and in providing experimentally testable hypotheses to characterize agonist-receptor interactions.

AChBP is homologous to the extracellular domain of the Cys-loop family of LGIC and therefore, given that there are as yet no atomic resolution structures of the latter proteins, homology modeling with AChBP provides a route to identifying important features of the extracellular domain. It should be remembered, however, that AChBP lacks many features of the complete receptor and therefore the limitations of such data must be borne in mind. For example, combining structural information from this protein with the highest available resolution images from the nACh receptor has revealed that in the absence of ACh, the extracellular domains of the α-(ligand-binding) subunits differ by rotations of their inner pore-facing parts when compared to the AChBP subunit structures (Unwin et al., 2002). As the structure of AChBP does, however, appear to provide a reasonable fit with the nACh receptor data in the presence of ACh, the use of the model we have derived to examine the docking of 5-HT should yield a sufficiently accurate estimation of the agonists' location to provide useful experimentally testable hypotheses. Indeed, the residues that we identified as <5 Å from 5-HT are all located in the binding loops A–E that were originally defined from photo-affinity labeling and mutagenesis studies of the related nACh receptor. These studies showed that residues contributing to the binding site were located in three regions of the α-subunit (loops A–C) and three regions on the adjacent β- or γ-subunit (loops D–F), although the latter appeared to play a less significant role. Consistent with these data, examination of the putative ligand-binding pocket of the 5-HT_3 receptor suggests that residues from the loop B and C regions play the major role, with fewer residues from loops A, D, and E being involved. Loop F has not yet been fully defined in the structure of AChBP, and thus we currently have no loop F residues in our model.

To identify which of the docking positions is most likely to represent the correct orientation of 5-HT in the binding site, we used both experimental data and evidence from sequence conservation. The latter is indicated in Table 1 and shows that 76% of the residues we identified to be within 5 Å of 5-HT are conserved in all agonist-binding 5-HT_3 receptor subunits identified to date. The only region that contains residues that are not conserved is loop C. As we would anticipate that critical residues would be conserved, models 4 and 5 are favored; here only two nonconserved residues are present in the binding site, as compared to four in the other models. Conversely, comparing those residues that are conserved but are not present in our defined binding site region, the most likely models are 4, 5, and 7 (one residue not present), whereas in the other models two residues are not present.

More definitive evidence, however, can be obtained from


1
T181
S182
Y234
N128
Y143
W183
5-HT
F226
Y153
Q151
I230
W90
I228
D229
R92
2
T181
L184
S182
Y234
N128
Y143
W183
5-HT
F226
Y153
I230
W90
I228
D229
R92
3
T181
L184
S182
Y234
N128
Y143
W183
F226
5-HT
Y153
Q151
I230
W90
I228
D229
R92
4
T181
L184
S182
Y234
T179
Y143
N128
E236
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Y153
Q151
W90
I230
I228
D229
R92
7
T181
L184
S182
Y234
Y143
E236
N128
W183
5-HT
F226
Y153
W90
I230
Q151
I228
D229
R92

TABLE 1 Residues of the putative 5-HT$_3$ receptor binding site within 5 Å of 5-HT

5-HT$_{3A}$		Model								α1 nAChR
Loop	Residue	1	2	3	4	5	6	7	CON	Residue
A	N128	+	+	+	+	+	+	+	y	Y93
B	T179	–	–	–	+	+	–	–	y	K145
	T181	+	+	+	+	+	+	+	y	G147
	S182	+	+	+	+	+	+	+	y	T148
	W183	+	+	+	+	+	+	+	y	W149
	L184	+	+	+	+	+	+	+	y	T150
C	F226	+	+	+	+	+	+	+	y	Y190
	I228	+	+	+	+	+	+	+	n	C192
	D229	+	+	+	–	–	+	+	n	C193
	I230	+	+	+	–	–	+	+	n	P194
	Y234	+	+	+	+	+	+	+	n*	Y198
	E236	–	–	–	+	+	–	+	y	D200
D	**W90**	+	+	+	+	+	+	+	y	R55
	R92	+	+	+	+	+	+	+	y	K57
E	**Y143**	+	+	+	+	+	+	+	y	L79
	Q151	+	+	+	–	–	+	+	y	T117
	Y153	+	+	+	+	+	+	+	y	T119

CON, conservation of this residue in all known 5-HT$_{3A}$ (agonist-binding) subunits to date. Bold indicates experimental evidence is available to support the role of this residue in the ligand-binding site. Equivalent residues in a nACh receptor subunit are shown for comparison.
*, conserved as aromatic.

experimental studies. The most useful of these are probably by the Lester lab, who have shown that the 1° amine of 5-HT forms a cation-π interaction with W183 in the binding site (Beene et al., 2001). Mutagenesis studies have previously shown that this tryptophan is a critical ligand-binding residue (Spier and Lummis, 2000), and it is homologous to W149 in the nACh receptor, which also is involved in a cation-π interaction with the agonist (Zhong et al., 1998). In other LGIC this residue also appears important; aromatic residues are found in the homologous position in GABA$_A$ and glycine receptors, where again they have been shown to play a role in ligand binding (Schmieden et al., 1993; Vandenberg et al., 1992; Amin and Weiss, 1993). These data favor models 4 and 5 and indeed allow us to dismiss models 6 and 7, where the aromatic center of tryptophan is not sufficiently close to the 1° amine of 5-HT to form such an interaction (Gallivan and Dougherty, 1999).

W183 is located in the loop B region. Our models suggest that up to four other residues from this region are also in the binding site: T181, S182, and L184 were observed in all models, and T179 in models 4 and 5. T179 is conserved not only in all agonist binding (A) subunits, but also in B and in the recently identified but as yet uncharacterized C subunits (Reeves and Lummis, 2002), but not in the nACh receptor or in AChBP. This suggests it may play an important role, further supporting models 4 and 5. Potential hydrogen bonds with some of these loop B residues also provide a useful experimentally testable point of distinction between these two models. In model 4, the backbone carboxyl group of S182 is an appropriate distance and orientation to form a hydrogen bond with primary amine of 5-HT, whereas in model 5 a hydrogen bond could form with the backbone carboxyl group of W183 (Fig. 3).

In loop A, the only residue within 5 Å of 5-HT is N128. This residue has not been previously identified as a ligand-binding residue, but interestingly the adjacent glutamate, E129, and also F130 have been proposed from mutagenesis studies to be involved in both agonist and antagonist binding (Steward et al., 2000). It is possible that changing these residues alters the location or orientation of N128. It is also possible, however, that our alignment may not be accurate in this region. Further mutational analysis will be required to distinguish between these possibilities.

Experimental evidence has also shown that loop C residues play a critical role in forming the binding site. Simultaneous mutagenesis of I228, D229, and I230 significantly changed the EC_{50} for 5-HT, although the single mutations have yet to be investigated (Hope et al., 1999). D229 is one of two acidic residues in the loop C region, and there is evidence that at least one of these is in close proximity to the ligand. The fluorescence profile of the 5-HT$_3$ receptor antagonist, GR-flu, is pH dependent, and when bound suggests an acidic character for the binding site (Tairi et al., 1998). Interestingly, D229, which is in the binding site in models 1, 2, 3, 6, and 7, is not conserved in all binding subunits. This is in contrast to E236, which is present in models 4, 5, and 7; thus, sequence data support the latter models. Y234 has also been shown in mutagenesis experiments to play a role at the binding site (Price and Lummis, 2001), perhaps in stabilizing the primary amine of 5-HT. In models 4 and 5 it is located at the opposite side of this amine group to W183 and may form another cation-π interaction here. Other mutagenesis studies of the loop C region also suggest that a number of these residues are involved in ligand binding; this region was found to be critical in controlling the potency of both the 5-HT$_3$ receptor agonist meta-chlorophenylbiguanide (Mochizuki et al., 1999) and the antagonist *d*-tubocurarine (Yan et al., 1999).

In loop D, two residues appear in all our models, W90 and R92, and there are experimental data from mutagenesis studies to support their role in the binding site (Mochizuki et al., 1999; Yan et al., 1999). These residues correspond to ligand-binding residues W53 and Q55 in the AChBP, which, as predicted by Yan et al. (1999) for the 5-HT$_3$ receptor, are part of a β-sheet structure in our model.

Two residues from loop E are also apparent in all our

FIGURE 3 Docking models for the binding of 5-HT to the 5-HT$_3$ receptor (1–7). Models 1–7, respectively, of 5-HT docked in the receptor binding site. Amino acids shown in each case project within a 5 Å radius of 5-HT. 5-HT is shown for clarity in a space-filling view, but these radii are not to scale. The protein is displayed in the same 3D orientation in each panel. Potential hydrogen bonds are shown as dotted green lines (lengths given in text). All other atoms are colored according to the CPK system (*blue*, nitrogen; *red*, oxygen; *white*, carbon).

models: Y143 and Y153. These data are also supported by mutagenesis studies that show changes in antagonist affinity when these tyrosines are replaced by serine or alanine (Price and Lummis, 2001). A separate systematic study of loop E of the 5-HT$_3$ receptor using radioligand binding also strongly implicates these tyrosines in ligand binding (Venkataraman et al., 2002). Q151 appears to be part of the binding site in models 1 and 6; however, there is currently no experimental evidence to support a role for this residue.

No residues from loop F are found in our models, as found in a ligand-docked model of the nACh receptor (Le Novère et al., 2002). As our model is not dynamic like the receptor itself, it may be that loop F plays a role in the binding site in states not represented by our model.

Models 1, 2, 3, 6, and 7 all display one potential hydrogen-bonding interaction with 5-HT, whereas models 4 and 5 have three positions for potential hydrogen bonds: with the backbone of S182 and the functional groups of E236 and N128. Not only would this favor tight binding of 5-HT in these two models, but also it is supported by an experimentally derived model of the pharmacophore for partial agonists (Daveu et al., 1999). This pharmacophore identifies two important hydrogen-bonding interactions at opposite ends of the ligand, which resemble those shown by models 4 and 5 described above. It is also interesting that these three potential hydrogen bonds are all in the one half of the cleft, which, according to the work of Unwin et al. (2002), would rotate on addition of agonist compared to the opposite side (which in our model is in the lower half, from residues W90 to Y143). Thus these hydrogen bonds could contribute to lowering the energy barrier for channel opening.

To date, two models of the nACh receptor binding site (Le Novère et al., 2002; Schapira et al., 2002) and one of the GABA$_A$ receptor binding site (Cromer et al., 2002) similarly based on the structure of AChBP have been published. Although many of the residues in the ligand-binding site are equivalent, the details differ significantly between the models. For example, Schapira et al. (2002) invoke a water molecule in the binding site between a hydrophilic pocket formed by loops B and C and the region of the ligands distal to the ammonium of ACh. This water is not found in a similar model (Le Novère et al., 2002). It is notable that both these models propose that the charged nitrogen of either ACh or nicotine intercalates between W148 and Y194. If the mechanisms of agonist binding are conserved between nACh and 5-HT$_3$ receptors, which seems a reasonable assumption, then we might expect that the charged nitrogen of 5-HT would be similarly located between a tryptophan and a tyrosine residue. This is indeed the case for models 4 and 5, and, therefore, assuming that one of these models is correct, it distinguishes the 5-HT binding mechanism of the ligand-gated 5-HT$_3$ receptor from that of the six known *metabotropic* 5-HT receptors. Here, models suggest that the indole group of 5-HT binds in a hydrophobic pocket formed by tryptophan and tyrosine residues, and that the charged primary amine is compensated by polar or charged residues, *not* via a cation-π interaction (Manivet et al., 2002). This supports the idea that metabotropic and ionotropic 5-HT receptors evolved independently, and therefore it is not surprising that different binding mechanisms exist for each receptor type.

Thus, overall, our data favor models 4 and 5. They are supported by both sequence-alignment data and experimental evidence. These two models only differ by a small rotation of 5-HT. However, as described above, the involvement of different hydrogen bonds in the two models should make distinguishing between them experimentally feasible. This ability to determine the accuracy of the models by experimentation is extremely important as has been demonstrated both by the production of two different models of the nACh receptor based on homology modeling, and by the demonstration that the ligand-binding subunits of the receptor may rotate on agonist binding. In addition, our models identify several residues, such as N128, S182, and E236, which may play important but as yet uncharacterized roles in ligand binding. Mutation of these residues will allow further refinement and a posteriori evaluation of the binding site models.

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