Chapter 3. Structure-function studies of tyrosines in the ligand-binding pocket of the 5-HT$_3$R

3.1 Introduction

The determination of the structure of the ACh binding protein (AChBP) which is homologous to the extracellular domain of the nACh receptor (nAChR) has had a significant impact on the study of ligand-gated ion channels (LGIC).[1] As the first high-resolution portrait of the extracellular domain of these proteins, it has provided crucial structural information for structure-function studies. In addition, lower resolution images from cryo-electron microscopy (cryo-EM) have contributed to our understanding of the global structure of these receptors.[2-6] Insights from both the AChBP and cryo-EM structures have led to a model of channel opening in which agonist binding induces a rotation of the extracellular domains. This is then thought to be transduced via the M2–M3 loop to M2, the pore-lining domain. The subsequent rotation of M2 removes the hydrophobic residues lining the channel and allows the passage of ions.[2] Although this is a plausible model, more experimental data are required both to substantiate it and to determine the molecular details of these processes.

One area where the AChBP structure has proven particularly valuable is in the generation of homology-based models. Homology models based on AChBP have been constructed for the nACh, GABA$_A$, and 5-HT$_3$ receptors.[7-10] The homology model of the 5-HT$_3$R reveals a ligand-binding pocket containing a large proportion of aromatic residues.[8] Two of these residues are tryptophans, one of which (Trp 183) forms a cation-π interaction with agonist.[11] There is also a phenylalanine (Phe 226), but most of

the aromatic character is provided by tyrosines (Tyr 141, Tyr 143, Tyr 153, and Tyr 234). The agonist-binding site in LGICs is composed of several non-contiguous loops (loops A-E). Tyrosines 141, 143, and 153 are located in loop E which is contributed to the binding site by the minus-side subunit (or in nAChR terms, the non-alpha subunit). This loop is not highly conserved in other members of the Cys-loop LGIC family. Tyr 234 is in loop C which is contributed by the plus-side subunit (or alpha-subunit in nAChR), and is universally conserved in the Cys-loop superfamily. Previous data have shown the importance of these residues in the binding and/or function of the 5-HT$_3$R, but it has not been possible to define their exact roles.[12, 13]

The work presented in this chapter combines conventional mutagenesis and nonsense suppression methodology to examine the functional roles of these binding-site tyrosines in the 5-HT$_3$R. This work represents a collaboration with the Sarah Lummis group of Cambridge University, and in particular Kerry Price, who performed all of the conventional mutagenesis experiments and binding studies. In addition she supplied significant contributions and effort to the nonsense suppression studies. It should also be noted that all of the modeling work considered here was generated by our Cambridge colleagues.

Using nonsense suppression, a series of tyrosine analogs was introduced at each tyrosine site (Figure 3.1).[14, 15] The tyrosine analogs were selected based on rational perturbations of tyrosine's physico-chemical properties. The precision afforded by unnatural amino acids provided key information for delineating the functional role of these tyrosines. Assimilating these data with the homology model of the 5-HT$_3$R binding site into which 5-HT has been computationally docked allowed us to confirm the

orientation of 5-HT in the agonist binding pocket and to determine several non-covalent interactions that are important for receptor binding and/or gating. This information was used to generate a possible mechanism by which agonist binding could trigger a series of conformational changes near the binding site that may initiate the gating process.
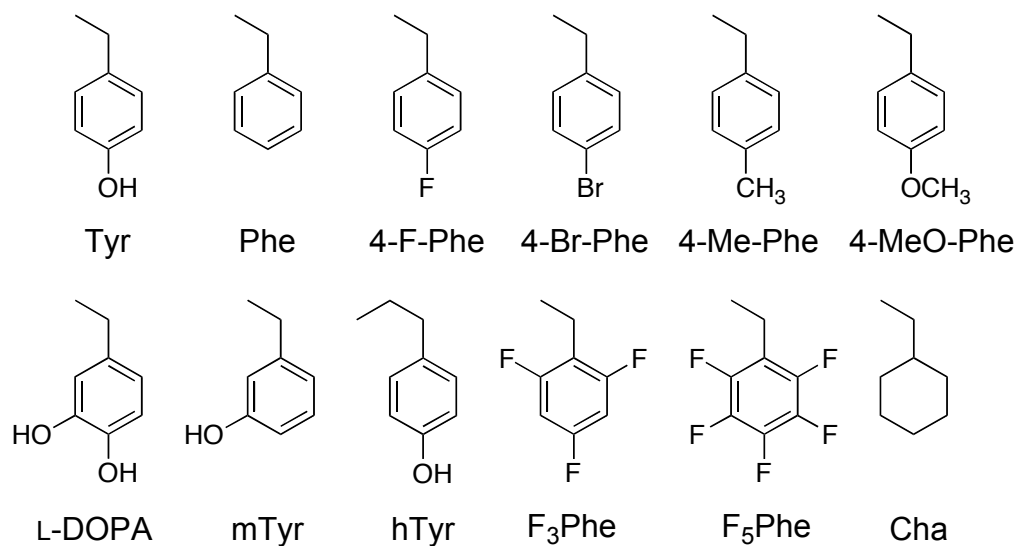

Figure 3.1. Structures of the side chains incorporated by nonsense suppression

3.2 Results

3.2.1 Nonsense suppression control experiments

3.2.1.1 Rescue of wild type through reintroduction of tyrosine

As an initial test of the viability of nonsense suppression at these sites, tyrosine was incorporated via acylated tRNA at each of the four positions (141, 143, 153, and 234). For the nonsense suppression methodology to give reliable results, the EC_{50} values and Hill coefficients for activation by 5-HT must replicate those of the wild-type (non-suppressed) $5\text{-HT}_{3A}R$, heterologously expressed in *Xenopus* oocytes.

All mutant mRNAs, when co-injected into *Xenopus* oocytes with tRNA-Tyr, produced functional receptors that responded to application of 5-HT with an inward current that desensitized in response to maintained 5-HT. The results shown in Tables 3.1 and 3.2 establish that the EC_{50} values and Hill coefficients obtained from 141-Tyr, 143-Tyr, 153-Tyr, and 234-Tyr and wild type receptors were indeed similar to each other. This indicates that the wild-type phenotype was successfully "rescued" by the delivery of tRNA-Tyr molecules.

3.2.1.2 *Validation of the pGEMHE vector*

One novel feature of these studies was the use of the pGEMHE vector.[16] Traditionally we have almost exclusively relied on the pAMV vector for nonsense suppression. This vector produces reliable expression and results, but is not the most amenable vector for subcloning, in that the start codon of the open-reading frame must be placed adjacent to the AMV sequence. This requirement limits the possible restriction enzymes for subcloning. The pGEMHE vector is a high expression oocyte vector and does not have such stringent subcloning requirements.

Suppression experiments using pGEMHE showed that maximal 5-HT-induced currents (I_{max}) were typically 0.8–2 μA at a holding potential of -60mV (Figure 3.2). This suggests that the pGEMHE vector can yield levels of full-length, correctly folded protein at least as high as the original pAMV vector.[15] The efficiency of nonsense suppression has been estimated at ~10 percent[14], and comparison of I_{max} values for the wild-type 5-HT$_{3A}$ receptor expressed in oocytes using pGEMHE to rescued TAG mutant receptors suggests a similar efficiency here.
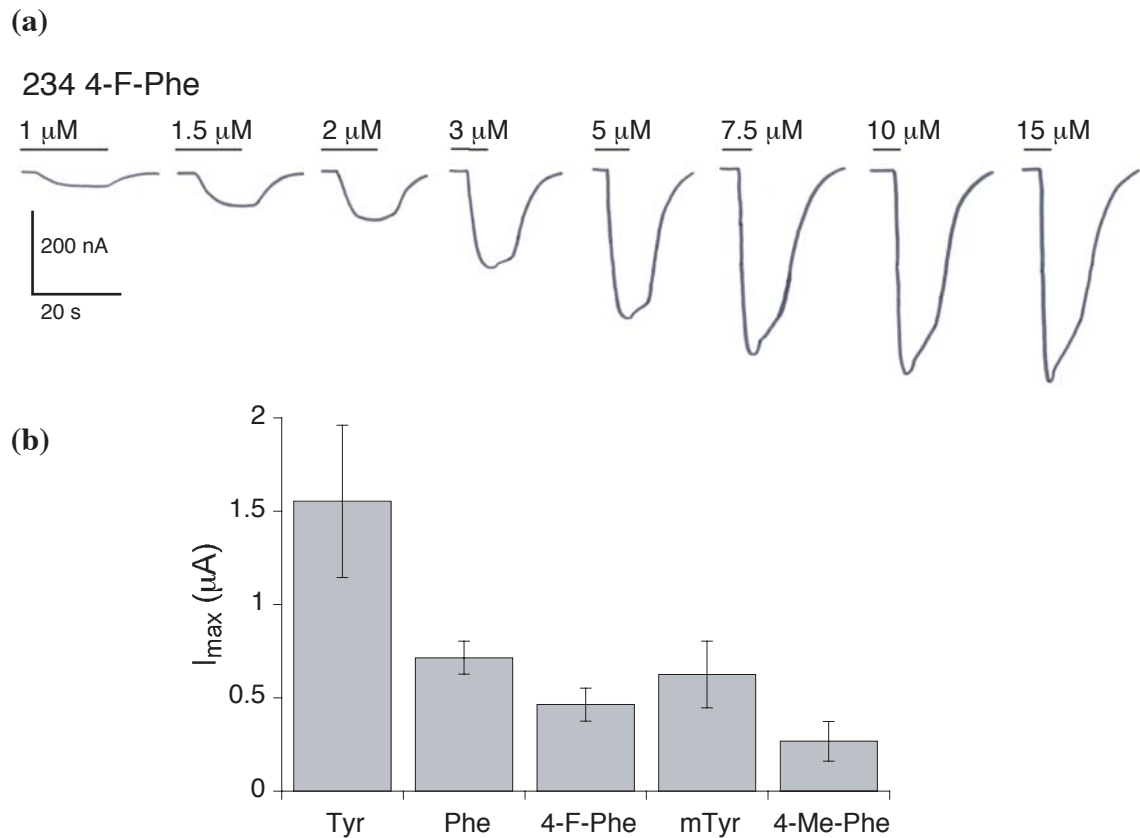
(a)

234 4-F-Phe

| 1 μM | 1.5 μM | 2 μM | 3 μM | 5 μM | 7.5 μM | 10 μM | 15 μM |

200 nA

20 s

(b)

I_{max} (μA)

Tyr   Phe   4-F-Phe   mTyr   4-Me-Phe

Figure 3.2.   (a) Representative voltage-clamp current traces for oocytes expressing suppressed 5-HT$_{3A}$R. Bars represent application of 5-HT. (b) Representaive maximal currents (I_{max}) for suppression experiments using the pGEMHE vector, obtained from 5-HT$_{3A}$R suppressed at postion 234.

3.1.1.3   Testing read-through

No currents in response to application of high concentrations of 5-HT (1mM) were detected from oocytes injected with mRNA alone or with mRNA and tRNA not ligated to dCA-aa (THG73 74-mer tRNA). It therefore appears that transcripts, which have been truncated by the inclusion of a stop codon at the position of the mutated tyrosine residue, cannot produce functional receptors. This is to be expected because these truncated receptors would contain none of the transmembrane regions required for ion channel activity. We can also conclude that any amino acid that is incorporated into

these four sites of the mutant receptor is specifically introduced by the injected tRNA-aa and not by the endogenous tRNA population of the oocyte. Finally, the lack of response from oocytes injected with mRNA and unacylated tRNA shows that there is no detectable reacylation of tRNA by endogenous synthetases.

3.1.2 Incorporation of unnatural amino acids

A subset of the unnatural amino acids shown in Figure 1 was selected for testing at each of the mutant positions. These compounds were previously incorporated into the nAChR and are therefore compatible with the oocyte expression system.[14, 15] In addition to the standard amino acids tyrosine and phenylalanine, the amino acids tested include: 3-hydroxyphenylalanine (meta-tyrosine or mTyr), homotyrosine (hTyr), L-DOPA, 4-methylphenylalanine (4-Me-Phe), 4-fluoro-phenylalanine (4-F-Phe), 2,4,6-F_3-phenylalanine (F_3-Phe), 2,3,4,5,6-F_5-phenylalanine (F_5-Phe), 4-methoxyphenylalanine (4-MeO-Phe), and cyclohexylalanine (Cha).

Current traces from a typical dose-response experiment (4-F-Phe at position 234) are shown in Figure 3.2. Although there were changes in the EC_{50} values and Hill coefficients for some mutant receptors (see below), activation and desensitization kinetics were similar to wild type for all receptors incorporating unnatural amino acids that responded to 5-HT.

3.1.3 Tyr 141

Incorporation of Phe, mTyr, 4-F-Phe, 4-MeO-Phe, F_5-Phe, and Cha using nonsense suppression mutagenesis and substitution by the natural amino acids Ala, Ser, and Phe using standard mutagenesis techniques produced receptors that responded to

5-HT in a manner very similar to wild-type 5-HT$_{3A}$R (Figure 3.3, Table 3.1 and Table 3.3). These data therefore show that neither the aromatic nor the hydroxyl groups are important for agonist binding or receptor gating. However, this residue has been implicated in antagonist binding because changes in affinity of 5-HT$_3$R antagonists have been observed when this residue was substituted with a non-aromatic amino acid.[12, 13] Thus, Tyr141Ala receptors have decreased [^{3}H]granisetron, D-tubocurarine, and lerisetron binding affinity compared with wild-type receptors, whereas Tyr141Ser receptors do not bind [^{3}H]granisetron at all. The model of the 5-HT$_3$R binding site (Figure 3.5) [based on model 4 of reference #8] supports these data, because Tyr 141 could contact these larger antagonists, but is not close enough to be in direct contact with a bound 5-HT molecule.[8]
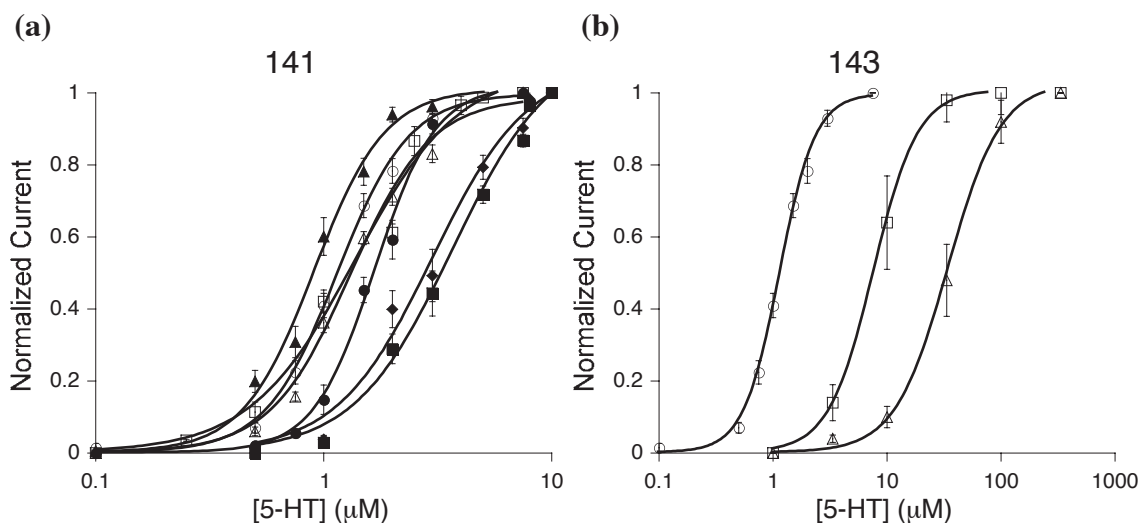

Figure 3.3. Dose-response relations for 5-HT$_{3A}$R nonsense suppression experiments. (a) Suppression at position 141: Tyr (*open circles*), mTyr (*filled circles*), 4-Me-Phe (*filled squares*), Phe (*filled triangles*), 4-F-Phe (*open triangles*), F$_5$Phe (*open squares*), and Cha (*filled diamonds*). (b) Suppression at position 143: Tyr (*open circles*), hTyr (*open triangles*), and DOPA (*open squares*).

In previous studies, Tyr141Ala and Tyr141Ser receptors did not function when expressed in human embryonic kidney 293 cells.[12, 13] However, our data show they have

similar characteristics to wild-type receptors when expressed in oocytes. Oocytes are generally more tolerant than mammalian cells to expression of ion channel proteins, which may require longer periods to fold correctly, the difference perhaps arising from the fact that oocytes are incubated at lower temperatures which would favor complex multisubunit assemblies.[17] These data therefore suggest a role for this residue in correct receptor folding and/or assembly, perhaps locally in the binding-site region.

3.1.4 Tyr 143

Data from Tyr 143 mutant receptors suggest that an aromatic ring with a hydroxyl group at the 4 position is essential for efficient receptor function. DOPA, which has these groups, was the only substitution at 143 that produced receptors with less than a 10-fold increase in EC_{50}. Incorporation of even subtle changes such as 4-F-Phe, 4-MeO-Phe, and mTyr resulted in receptors that did not respond to 5-HT (Table 3.1). DOPA also has a hydroxyl at the 3 position, and the increase in EC_{50} observed with this compound (approximately sixfold) suggests that bulk here is not desirable. An increased chain length or removal of the hydroxyl, however, is more deleterious. Phe and hTyr caused 30- to 40-fold increases in EC_{50}. Removal of the aromatic group and/or further displacement of the hydroxyl group as in replacement with Ala or Ser resulted in even more severe changes: 100-fold EC_{50} increases. In addition, changes in apparent activation and desensitization rates (10–90% rise times were twofold to fourfold slower than wild-type receptors) (Table 3.3), and little or no desensitization were observed. These data demonstrate the importance of both the aromatic and the hydroxyl group of Tyr 143, and in particular, suggest that the hydroxyl forms a hydrogen bond that is essential for efficient receptor gating.

To confirm the presence of such a bond would require x-ray crystal structural data which unfortunately, is not available. However, mutagenesis data alone can provide strong evidence, particularly when there is some supporting structural data such as a homology model.[18] Indeed, the unnatural amino acid methodology employed here is a much more powerful probe for this type of bond than conventional mutagenesis because not only can we introduce subtle changes that minimally perturb the global protein structure, but also design the amino acid to determine whether the residue acts as a hydrogen bond donor or acceptor. Thus, using conventional mutagenesis, we can remove the hydroxyl at position 143 (in Phe), but not replace it. Ser is unsatisfactory as a replacement because hydrogen bonds depend critically on the distance between donor and acceptor atoms, and the hydroxyls of Ser and Tyr are in quite distinct locations. Unnatural amino acid substitution can surmount this problem; DOPA has a similarly placed hydroxyl to Tyr, whereas 4-MeO-Phe places an oxygen atom in the same location, and the hydroxyl of hTyr is only subtly displaced from that of Tyr. Data from these substitutions support the proposal of a hydrogen bond at Tyr 143 and indeed, further suggest that the hydroxyl here acts as a hydrogen bond donor.

The model of 5-HT docked into the 5-HT$_{3A}$R extracellular domain (Figure 3.5) shows that the hydroxyl of Tyr 143 has the potential to hydrogen bond with one of a number of residues on the opposite side of the binding pocket. These are the hydroxyl or the ring of Tyr 234 and the carbonyl oxygen of Trp 183 or Leu 184, all of which are 3Å away. The fact that 4-Me-Phe could substitute satisfactorily for Tyr 234 suggests that there is no such bond between the hydroxyls of Tyr 143 and Tyr 234, but as yet, we cannot exclude any of the other possibilities. Of course, the accuracy of the inter-residue

distances must be viewed with caution both because of the problems inherent with using a model based on a homologous structure, and because in the docking procedure, the protein side chains remain rigid, which is certainly not the case in the functioning receptor. Nevertheless, the modeling data suggest that the most probable hydrogen bonding partner of Tyr 143 is the backbone carbonyl of Trp 183 — an interaction that has also been previously suggested.[19]

The presence of either an aromatic or a hydroxyl at position 143, however, is not essential for antagonist interactions: alanine, serine, and phenylalanine mutants of Tyr 143 bound [³H]granisetron with an affinity similar to wild-type receptors (Table 3.3).[12, 13] Competitive antagonists such as granisetron bind in the binding pocket, but cannot trigger the conversion to the open state. These data, combined with the modeling data, thus suggest that neither an aromatic group nor a hydrogen bond is required for ligand binding, but both are essential for efficient receptor gating.

3.1.5   Tyr 153

Large increases in EC_{50} values, modifications in receptor kinetics, and changes in [³H]granisetron binding affinity (Figure 3.4, Table 3.2 and Table 3.3) in mutant receptors suggest that Tyr 153 plays a role in both binding and gating of the receptor. The data show that both an aromatic ring and a hydroxyl group in the 4 position are required for correct receptor function. Removing (Ala, Ser) or drastically perturbing (F$_5$Phe) the aromatic ring resulted in 100-fold increases in EC_{50},. Deleting the tyrosine hydroxyl group (Phe) or replacing it (4-F-Phe or 4-Me-Phe) led to ~20-fold increases in EC_{50}, and relocating it (mTyr and Ser) resulted in 100-fold EC_{50} increases. However, 4-MeO-Phe increased EC_{50} only sixfold, indicating that the importance of the hydroxyl is via its

oxygen atom. These data strongly indicate the presence of a hydrogen bond with the hydroxyl of Tyr153 functioning as a hydrogen bond acceptor.

Table 3.1   Dose-response data for suppression at positions 141 and 143

Residue	141		143	
	$EC_{50} \pm SEM$ (μM)	Hill±SEM	$EC_{50} \pm SEM$ (μM)	Hill±SEM
Tyr	1.16 ± 0.04	2.76 ± 0.20	1.15 ± 0.03	2.98 ± 0.19
Phe	0.92 ± 0.06	2.80 ± 0.44	78.6 ± 2.86	2.73 ± 0.21
4-MeO-Phe	3.36 ± 0.14	2.17 ± 0.16	NR	NR
4-F-Phe	1.32 ± 0.05	2.47 ± 0.17	NR	NR
F_5-Phe	1.38 ± 0.08	1.89 ± 0.17	NA	NA
Cha	3.14 ± 0.40	1.92 ± 0.21	NA	NA
mTyr	1.69 ± 0.06	3.24 ± 0.29	NR	NR
hTyr	NA	NA	33.24 ± 2.60	1.80 ± 0.22
DOPA	NA	NA	7.16 ± 0.90	2.34 ± 0.54

NA = not attempted, NR = no response to concentrations up to 1 mM 5-HT

Table 3.2   Dose-response data for suppression at positions 153 and 234

Residue	153		234	
	$EC_{50} \pm SEM$ (μM)	Hill±SEM	$EC_{50} \pm SEM$ (μM)	Hill±SEM
Tyr	1.15 ± 0.04	2.78 ± 0.20	1.24 ± 0.05	2.89 ± 0.28
Phe	22.5 ± 0.43	2.60 ± 0.11	10.22 ± 0.80	1.92 ± 0.26
4-MeO-Phe	6.65 ± 0.27	1.98 ± 0.12	NA	NA
4-F-Phe	19.7 ± 1.22	1.82 ± 0.14	4.11 ± 0.20	2.36 ± 0.19
4-Br-Phe	NA	NA	1.78 ± 0.08	2.79 ± 0.32
4-Me-Phe	18.2 ± 0.48	2.48 ± 0.11	1.13 ± 0.05	2.49 ± 0.22
F_3-Phe	NA	NA	NR	NR
F_5-Phe	(>500)		NR	NR
Cha	NA	NA	NR	NR
mTyr	(>500)		7.42 ± 0.32	2.83 ± 0.32

NA = not attempted, NR = no response to concentrations up to 1 mM 5-HT

In the homology model, this hydroxyl is located such that it could participate in a hydrogen bond interaction, either with the 5-HT indole nitrogen (2.9 Å away) or with Arg 92 (3.1 Å). Arg 92— like Tyr 153— is located on the subunit "inner loop," which is

proposed to move relative to the "outer loop" after receptor activation.[3] Because the

relative movement of the two residues during channel opening would therefore be

negligible, it is unlikely that removing a hydrogen bond here would result in the changes

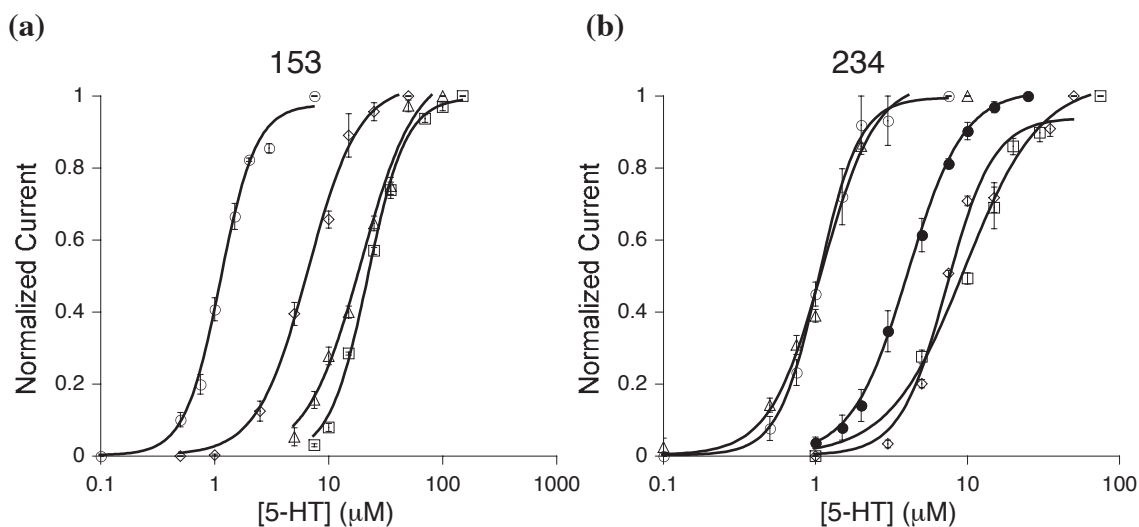that we observed. It therefore seems plausible that Tyr 153 hydrogen bonds to 5-HT.

(a) **(b)**

Figure 3.4. Dose-response relations for 5-HT$_{3A}$R nonsense suppression
experiments. (a) Suppression at position 153: Tyr (*open circles*), Phe (*open squares*),
4-F-Phe (*open triangles*), and 4-MeO-Phe (*open diamonds*). (b) Suppression at position
234: Tyr (*open circles*), mTyr (*open diamonds*), Phe (*open squares*). 4-F-Phe (*filled circles*),
and 4-Me-Phe (*open triangles*).

3.1.6 Tyr 234

Replacement of tyrosine at this position with Phe, 4-F-Phe, mTyr, and 4-Me-Phe

produced receptors for which EC$_{50}$ values for 5-HT activation were either unchanged or

increased only 10-fold compared with wild-type receptors (Figure 3.4, Table 3.2). Thus,

the hydroxyl group of Tyr 234 is not essential for correct receptor function. Indeed, the

equivalent residue here in the guinea pig 5-HT$_3$R is Phe.[20] The electrostatic potential of

the aromatic group here, however, does seem important. Replacement with Cha, Ala or

Ser resulted in nonfunctional receptors. In many ways Cha is an ideal mutation for

examining the importance of aromatic character. It is nearly isosteric with Phe and maintains very similar shape and hydrophobicity, but cannot participate in any of the special aromatic interactions (cation-π, CH-π, or aromatic-aromatic). Thus, its non-viability at this site does suggest a role for the aromatic character of Tyr.

The potential importance of the aromatic group is supported by the results with 4-F-Phe and F_5-Phe. Here, a single fluorine substituent (4-F-Phe) caused a small increase in EC_{50}, and multiple fluorine substituents (F_5-Phe) resulted in nonfunctional receptors. Substitution of the aromatic group with electron-withdrawing groups pulls electron density away from the face of the aromatic ring, thereby disrupting the special aromatic interactions listed above. The data, however, are not consistent with a cation-π interaction at 234. Phe— which displays a similar affinity as Tyr in binding cations— leads to an eightfold increase in EC_{50}, but 4-F-Phe and 4-Br-Phe— both of which would almost equally weaken any cation-π interaction, lead to roughly 1.5- and 3-fold increases, respectively in EC_{50}. These results are not consistent with a cation-π interaction at 234, which is interesting because in the model (Figure 3.5), this residue has the potential to interact with the primary ammonium of 5-HT. Thus, these results provide experimental feedback for improving the homology model.

The pattern of EC_{50} values for unnatural amino acid substitution at 234 is consistent with bulk at the 4 position of the aromatic ring being required for correct receptor function. Those residues with a substituent at the 4 position (4-Me-Phe, 4-Br-Phe, and 4-F-Phe) give lower EC_{50} values than those without (Phe, mTyr). Such a finding was reported for the aligning residue (Tyr198) in the nAChR[21], where a larger number of unnatural residues were tested. The size of this substituent also appeared to be

important, with larger substituents not being tolerated and smaller ones also being less

favored, suggesting a steric role.

Table 3.3   Conventional mutagenesis studies: dose-response data,
[³H]granisetron binding affinities, and 10-90% rise times for current onset

Residue	EC$_{50}$±SEM (µM)	Hill±SEM	K$_d$ (nM)	Rise time (s)
Wild type (Y)	1.34 ± 0.12	2.38 ± 0.23	0.32 ± 0.035	2.1 ± 0.3
141				
Ala	2.73 ± 0.15	2.68 ± 0.4	8.97 ± 2.44	2.4 ± 0.2
Ser	4.7 ± 0.38	2.02 ± 0.31	NB	2.4 ± 0.3
Phe	ND	ND	0.98 ± 0.15	ND
143				
Ala	354 ± 29.0	2.62 ± 0.53	1.2 ± 0.24	7.9 ± 0.5
Ser	472 ± 47.9	2.68 ± 0.62	1.1 ± 0.23	8.4 ± 0.8
Phe	78.6 ± 2.86	2.73 ± 0.21	0.53 ± 0.10	4.5 ±1.0
153				
Ala	120 ± 9.43	2.43 ± 0.38	3.62 ± 1.75	8.3 ± 0.7
Ser	84.1 ± 5.60	2.54 ± 0.37	NB	8.1 ± 0.4
Phe	ND	ND	0.53 ± 0.10	ND

ND = No data, NB = No binding

3.1.7   A new model of the 5-HT binding pocket

The new data strongly support previous suggestions by the Lummis group arising

from their modeling studies that the correct orientation of 5-HT in the binding pocket is

with the ammonium group located between Trp 183 and Tyr 234. In addition, the data

are consistent with hydrogen bonds between Tyr 143 and Trp 183, and Tyr 153 and 5-HT

(Figure 3.5). The Lummis group also created a new "closed state" model based on the

structural work by Unwin and coworkers.[3] Unwin's data indicate differences in the

binding pocket between the closed state of the nAChR and AChBP (which is considered

to be closer to the open or desensitized state). In the closed state model, Tyr 143 and 153

are 3 Å closer to the center of the pocket, and clashes between these residues and 5-HT

indicate that binding of this agonist in the orientation supported by the experimental data is not possible (Figure 3.6). Antagonist binding, however, would not be affected. Thus, if we assume that similar movements in the binding pocket occur in the nACh and 5-HT$_3$ receptors, and that the new model provides a reasonable approximation of the closed state of the receptor, then Tyrs 143 and 153 would need to be displaced to allow 5-HT to dock into the binding site (Figure 3.6).

3.3   Discussion

The *in vivo* nonsense suppression method of unnatural amino acid incorporation is a powerful tool for the investigation of receptor structure-function relationships. Using a selection of tyrosine analogs, we show that tyrosines located in or close to the receptor binding pocket each play a different role in receptor function and do not simply provide a featureless extended aromatic environment. Tyr 141 does not appear to be a critical site, as the various mutations introduced here had little effect. Tyr 143 and Tyr 153, however, are important sites for receptor function, and the results for substitution at these sites indicate that the hydroxyls of these tyrosines form functionally necessary hydrogen bonds. At Tyr 234 we find that both the aromatic group and an appropriately sized substituent at the 4 position of the ring are important. These data, combined with previous work from the Dougherty group elucidating the cation-π interaction between Trp 183 and the ammonium group of 5-HT[11], have provided insight into the correct orientation of 5-HT in a homology model of the 5-HT$_3$R binding site. In addition, findings from the present study, along with a new closed state model of the 5-HT$_3$R, suggest a possible series of bond rearrangements in the binding site that are required for

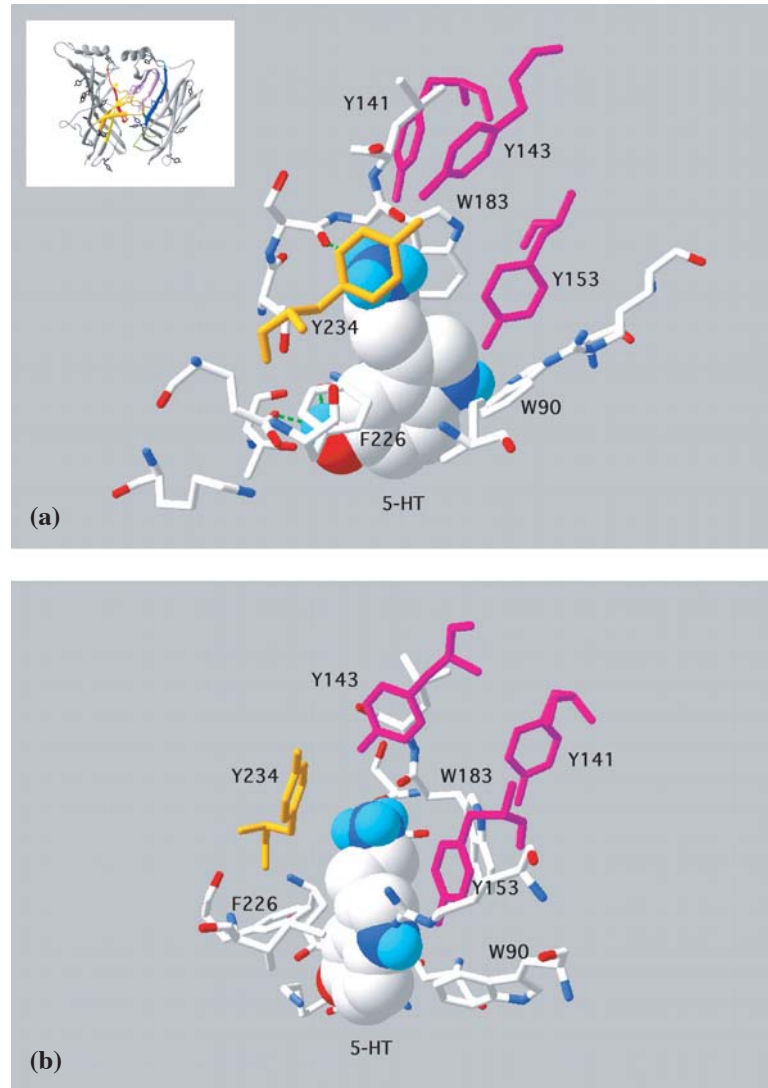gating, and thus may represent early conformational changes that lead to channel opening.

Figure 3.5.   Model showing two views of 5-HT docked into the 5-HT3A receptor binding site. This is based on model 4 of reference #8 (Reeves et al. (2003)). Tyrosine residues considered here are shown in purple for loop E (141, 143, 153) and orange for loop C (234,). Docked 5-HT is shown in CPK, with the primary ammonium group sandwiched between Trp183 where there is a cation- interaction and Tyr234. The indole nitrogen abuts the Tyr153 side chain. Other binding site residues are labeled.

3.1.1   Roles of 5-HT$_3$R binding-site tyrosine residues

Tyr 141 does not play a critical role in 5-HT$_3$R agonist binding or gating, but it may be involved in antagonist binding and receptor assembly. The recent structure of AChBP bound to agonists shows that the residue equivalent to Tyr141 (Leu 102) hydrogen bonds to nicotine via a water molecule.[22] In the homology model of the 5-HT$_3$R, Tyr 141 does not contact 5-HT (and indeed in AChBP, residue Leu 102 does not contact carbachol), but this does not preclude from it interacting with larger antagonists and/or to another residue in the binding pocket, perhaps during subunit folding to assist its correct formation.

Tyr 143 is a sensitive residue in that it can only be replaced by a limited number of alternative amino acids to form functional receptors. Data from these mutant receptors (no change in [^{3}H]granisetron binding affinity and a large change in EC$_{50}$) combined with the modeling data (no interaction with 5-HT) strongly suggest that Tyr 143 forms a hydrogen bond between two regions of the receptor that is essential for receptor gating. The modeling data further suggest that this bond may be between the hydroxyl group and the backbone carbonyl group of Trp 183, which has been previously suggested by Maksay et al.[19] Previous data have also shown the importance of this residue in 5-HT$_3$R function[13], and the equivalent residue in AChBP (Arg 104) has been shown to make contact with carbachol.[22]
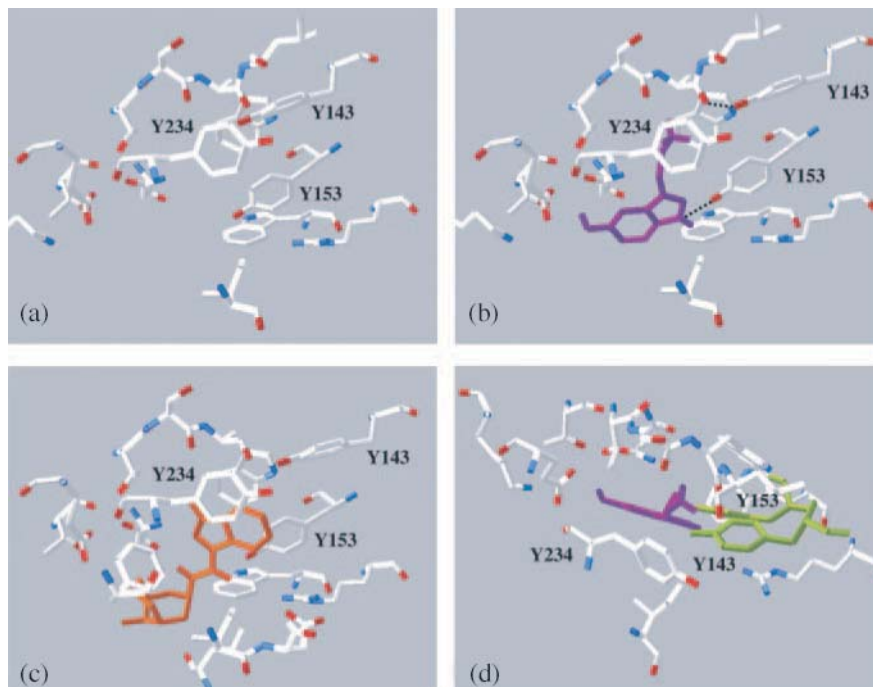
Figure 3.6. (a) Model of the 5-HT$_3$R binding site in the closed state; Tyr 143 and Tyr 153 are 3 Å closer to the center of the pocket than in the open state model. (b) Model of the 5-HT$_3$R binding site in the open state with 5-HT (purple) docked, showing potential new hydrogen bonds formed by Tyr143 and Tyr153. (c) Model of the 5-HT$_3$R binding site in the open state with granisetron (orange). Granisetron, however, is most likely to bind to the closed state and, thus, there may be some inaccuracies in this model. Nevertheless, granisetron in this orientation would fit into the closed state model, where it would be within 3Å of Tyr 153 but further from Tyr 143. (d) Rotated model *a* with 5-HT (purple) docked in the same position as in *b*. Steric interference with Tyr 143 and Tyr153 (both green) would not allow 5-HT to be docked in this position.

The data also strongly suggest that Tyr 153 forms a hydrogen bond, and that this bond plays a role in both binding and gating. The equivalent residue in AChBP (Met 114) makes contact with both carbachol and nicotine[22], and data from other studies also suggest that it is involved in gating. Tyr153Ala mutant receptors display unusual response and desensitization kinetics[13], and mutation of the aligning residue in the GABA$_A$ receptor α-subunit (Thr 142) resulted in the antagonist flumazenil acting as a partial agonist.[23] Interestingly, the equivalent residue in the 5-HT$_{3B}$ subunit is histidine.[24]

Thus, heteromeric (A plus B) receptors would also have a residue with the potential to hydrogen bond in the binding site at this position.

Thus, our data show that the hydrogen bonds formed by Tyr 143 and Tyr 153 are critical for correct receptor function, suggesting these bonds play an important role in the conformational change leading to gating. This could be because they stabilize the bound state of the receptor and/or provide the energy required for protein rearrangement.

An aromatic residue at position 234 is essential for 5-HT$_3$R function. Indeed, conservation of an aromatic residue at the aligning position among all 5-HT$_3$R subunits— and in fact all Cys-loop receptors— indicates the importance of this aromatic group which appears to play a vital role as part of the "aromatic box" proposed to be critical for agonist binding in all members of this LGIC family.[25] However, a substituent at the 4 position appears to be important solely for gating because antagonist binding is unaffected by removal of the Tyr 234 hydroxyl, and the docking data suggest that there is no interaction between this group and 5-HT.[8] The effect is steric because hydroxyl and bromine which have similar sizes yield optimum function, whereas smaller substituents such as fluorine and hydrogen are less effective.

3.1.2   A model for initiating gating

The differences observed between AChBP x-ray crystal data and nAChR cryo-electron microscopy data suggest that AChBP better represents the open rather than the closed state of the receptor.[3] Thus, the docking of 5-HT into the homology model of the 5-HT$_3$R binding site as shown in Figure 3.5 is likely to be broadly accurate, whereas removal of 5-HT from this structure would not be a good representation of the closed state. Data from Unwin and coworkers show that in this region, the residues

equivalent to Tyr 143 and Tyr 153 are closer to the residues equivalent to Trp 183 and Tyr 234 in the unbound (closed state) nAChR compared with AChBP (open-like state).[3] These changes in distance are likely to be only a few ångstroms, because the binding site is close to the point around which the inner and outer loops of the subunit pivot. Shown in Figure 3.6 is a homology model of the 5-HT$_3$R binding site in the closed state based on Unwin's data. Here, both Tyr 143 and Tyr 153 are 3 Å closer to the center of the binding pocket. We propose that 5-HT entering the pocket forms a cation-π interaction with Trp 183 and in so doing, displaces Tyr 143. The hydroxyl of Tyr 234 ensures that Tyr 143 moves toward and subsequently, hydrogen bonds with the backbone carbonyl of Trp 183 and not with another E loop residue, as might be the case if formation of the alternative rotamer was not prevented by the 234 hydroxyl group. Tyr 153 is also relocated as 5-HT enters the pocket, assisted by the hydrogen bond it forms with the indole amine on 5-HT. These two residues (Tyr 143 and Tyr 153) are located on separate β-sheets linked by a turn and therefore, their combined movement could provide considerable torsional force. Combined with energy provided by the formation of hydrogen and other bonds, this could initiate the twist that triggers the conformational change.

Some support for this hypothesis comes from docking studies with the 5-HT$_3$R antagonist granisetron, which when bound does not induce the conformational changes associated with receptor gating. A recent report by Maksay and coworkers suggests that granisetron docked into a homology model of the 5-HT$_3$R binding site does not approach Tyr 143, but does come relatively close to Tyr 153 and interacts strongly with the aromatic group of Tyr 234.[19] Docking studies performed by the Lummis group (unpublished observations) similarly reveal potential interactions of granisetron with

Tyr 234 and Tyr 153, but not with Tyr 143. One orientation of granisetron that was observed, and is similar to that reported by Maksay and coworkers is shown in Figure 3.5. Granisetron in this orientation would also fit comfortably into the closed state model, and indeed, it may hydrogen bond with the hydroxyl of Tyr 153 (3 Å distant) in this model.

Additional support comes from reports of movement in the nAChR binding site[2, 3, 26] and in the $GABA_A$ receptor[27], which is likely to be similar in all Cys-loop receptors. However this has not yet been confirmed as no ligand–free atomic resolution structure of AChBP has yet been resolved.

3.4   Conclusion

In summary, the data obtained from introducing subtle changes to tyrosine residues in the $5\text{-}HT_3R$ binding site— combined with those obtained from models of the binding pocket— have shown distinct roles of each of these residues in the binding site. Combining these data with those from a model of the binding pocket has allowed us to generate a hypothesis of the mechanism that triggers the conformational change leading to channel opening. The data therefore show the power of unnatural amino acid mutagenesis in providing high-precision information that is highly complementary to modeling efforts. In addition, given the structural and functional similarity of ligand-gated ion channels, we believe the proposed mechanism of conformation change (agonist-stimulated movement of the binding loops combined with the formation of novel hydrogen bonds) will be broadly similar in all members of the family.

3.5 Methods

3.5.1 Mutagenesis and preparation of cRNA and Oocytes

Mutant $5\text{-HT}_{3A}R$ subunits were developed using the eukaryotic expression vector, pcDNA 3.1 (InVitrogen, Abingdon, U.K.), containing the complete coding sequence for the $5\text{-HT}_{3A(B)}$ subunit from NIE-115 cells as previously described.[28] Mutagenesis reactions were performed using the Kunkel method[29] and confirmed by DNA sequencing. Wild type (WT) and mutant receptor subunit coding sequences were then subcloned into pGEMHE plasmid.[16] This was linearized with Nhe1 (New England Biolabs) and cRNA synthesized using T7 mMESSAGE mMACHINE kit (Ambion). Oocytes from *Xenopus laevis* were prepared and maintained as described previously.[11]

3.5.2 Synthesis of tRNA and dCA-amino acids

Unnatural amino acids were chemically synthesized as nitroveratryloxycarbonyl (NVOC) protected cyanomethyl esters and coupled to the dinucleotide dCA, which was then enzymatically ligated to 74-mer THG73 tRNA_{CUA} as detailed previously.[30] Immediately prior to co-injection with mRNA, tRNA-aa was deprotected by photolysis.[31] Typically, 5 ng mRNA and 25 ng tRNA-aa were injected into Stage V-VI oocytes in a total volume of 50 nl. For control experiments, mRNA was injected 1) in the absence of tRNA and 2) with the THG73 74-mer tRNA. Experiments were preformed 18-36 h post injection.

3.5.3 Characterization of mutant receptors

5-HT-induced currents were recorded from individual oocytes using two-electrode voltage clamp with either a GeneClamp 500 amplifier or an OpusXpress system

(Axon Instruments, Inc., Union City, CA). All experiments were performed at 22-25° C.

Serotonin (creatinine sulphate complex, Sigma) was stored as 25 mM aliquots at -80°C,

diluted in calcium-free ND96, and delivered to cells via computer-controlled perfusion

systems. Glass microelectrodes were backfilled with 3 M KCl and had a resistance of

approximately 1 MΩ. The holding potential was -60 mV unless otherwise specified. To

determine EC_{50}s, 5-HT concentration-response data were fitted to the Hill equation,

$I = (I_{max}[A]^n)/(EC_{50}{}^n + [A]^n)$, where I_{max} is the maximal peak current, $[A]$ is the

concentration of agonist, and n is the Hill coefficient.

3.6   References

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