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Computational Model of the Complex between GR113808 and the 5-HT$_4$ Receptor Guided by Site-Directed Mutagenesis and the Crystal Structure of Rhodopsin *

María L. López-Rodríguez[b], Marta Murcia[b], Bellinda Benhamú[b], Mireia Olivella[a], Mercedes Campillo[a] & Leonardo Pardo[a,*]
[a]*Laboratori de Medicina Computacional, Unitat de Bioestadística, Facultat de Medicina, Universitat Autònoma de Barcelona, E-08193 Bellaterra, Spain;* [b]*Departamento de Química Orgánica I, Facultad de Ciencias Químicas, Universidad Complutense, E-28040 Madrid, Spain*

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Summary

A computational model of the transmembrane domain of the human 5-HT$_4$ receptor complexed with the GR113808 antagonist was constructed from the crystal structure of rhodopsin and the putative residues of the ligand-binding site, experimentally determined by site-directed mutagenesis. The recognition mode of GR113808 consist of: (i) the ionic interaction between the protonated amine and Asp$^{3.32}$; (ii) the hydrogen bond between the carbonylic oxygen and Ser$^{5.43}$; (iii) the hydrogen bond between the ether oxygen and Asn$^{6.55}$; (iv) the hydrogen bond between the C-H groups adjacent to the protonated piperidine nitrogen and the π electrons of Phe$^{6.51}$; and (v) the π-σ aromatic-aromatic interaction between the indole ring and Phe$^{6.52}$.

This computational model offers structural indications about the role of Asp$^{3.32}$, Ser$^{5.43}$, Phe$^{6.51}$, Phe$^{6.52}$, and Asn$^{6.55}$ in the experimental binding affinities. Asp$^{3.32}$Asn mutation does not affect the binding of GR113808 because the loss of binding affinity from an ion pair to a charged hydrogen bond is compensated by the larger energetical penalty of Asp to disrupt its side chain environment in the ligand-free form, and the larger interaction between Phe$^{6.51}$ and the piperidine ring of the ligand in the mutant receptor. In the Phe$^{6.52}$Val mutant the indole ring of the ligand replaces the interaction with Phe$^{6.52}$ by a similarly intense interaction with Tyr$^{5.38}$, with no significant effect in the binding of GR113808. The mutation of Asn$^{6.55}$ to Leu replaces the hydrogen bond of the ether oxygen of the ligand from Asn$^{6.55}$ to Cys$^{5.42}$, with a decrease of binding affinity that approximately equals the free energy difference between the SH$\cdots$O and NH$\cdots$O hydrogen bonds.

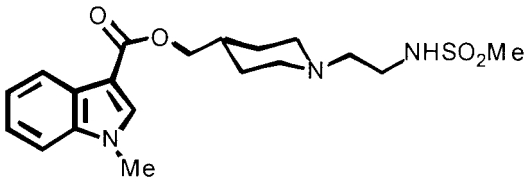
Because these residues are also present in the other members of the neurotransmitter family of G protein-coupled receptors, these findings will also serve for our understanding of the binding of related ligands to their cognate receptors.

Abbreviations: 5-HT$_4$R, 5-HT$_4$ receptor; GPCR, G protein-coupled receptor; TM, transmembrane helix; RHO, rhodopsin

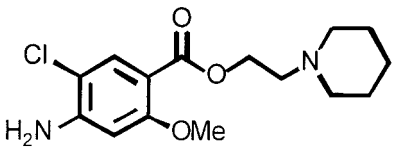
*To whom correspondence should be addressed. E-mail: Leonardo.Pardo@uab.es

Introduction

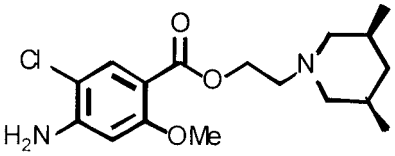
The 5-HT_4 receptor (5-HT_4R) belongs to the G protein-coupled receptor (GPCR) superfamily that transmit extracellular signals of neurotransmitters, peptides and glycoproteins through heterotrimeric G proteins bound in the interior of the cell [1]. The 5-HT_4R is of considerable interest because it is involved in (patho)physiological processes both in peripheral and central nervous systems [2]. A major advance in search for more potent and selective 5-HT_4R antagonists came with the identification of GR113808 [[1-[2-(methylsulphonylamino)ethyl]-4-piperidinyl]methyl-1-methyl-1H-indole-3-carboxylate], a highly potent and competitive antagonist of the 5-HT_4R [3]. GR113808 behaved as an antagonist of serotonin in guinea pig ascending colon ($pA_2 = 9.2$), rat oesophagus ($pA_2 = 9.5$), and human atrium ($pK_b = 8.8$). GR113808 is also highly selective with only weak affinity at 5-HT_3 receptors ($pK_i = 6.0$) and no activity at other 5-HT receptors (up to 10 μM). GR113808 was subsequently tritiated and it is normally used in both binding assays and radiographic analysis [2, 4].



GR113808



ML10302



ML10375

The modification of the amino acid sequence of members of the GPCR family of receptors, using methods of molecular biology, is a common procedure to define the amino acid side chains of the receptor that form the ligand binding pocket [5]. Recently, the binding site of serotonin, GR113808, ML10302 [6], and ML10375 [6, 7] to the human 5-HT_4R has been explored by site-directed mutagenesis [8]. Serotonin anchors the completely conserved $Asp^{3.32}$ (see Methods for receptor-numbering scheme), in transmembrane helix 3 (TM 3), throughout its protonated amine, as revealed by the lack of binding affinity of serotonin to the $Asp^{3.32}$Asn point mutation [8]. Surprisingly, the antagonist GR113808 is not influenced by this mutation, the agonist ML10302 is only weakly affected, and the antagonist ML10375 is moderately affected; despite all these compounds contain a protonated amine moiety. These results are in contrast to the observation that $Asp^{3.32}$ binds both agonists and antagonists (see [5] for a review), in the other members of the neurotransmitter family of receptors. On the other hand, substitution of $Ser^{5.43}$, in TM 5, by Ala avoids the binding of GR113808 [8]. TM 5 possesses, in the neurotransmitter family of receptors, a series of conserved Ser/Thr residues, at positions 5.43 and 5.46, that appear to hydrogen bond the hydroxyl groups present in the chemical structure of many neurotransmitters [9]. Thus, it was reasonably hypothesized that the hydroxyl group of serotonin and the carbonyl oxygen of the ester group of GR113808 are involved in the hydrogen bond to $Ser^{5.43}$. It has recently been shown that another Ser residue at position 5.42 in the β_2-adrenergic receptor is also involved in the binding of catecholamine ligands [10]. Both $Ser^{5.42}$ and $Ser^{5.43}$ of the β_2-adrenergic receptor interact with the meta-hydroxyl group of catecholamine ligands [10]. The 5-HT_4R contains a Cys residue at this 5.42 position. Substitution of $Cys^{5.42}$ by Ala in the 5-HT_4R increases the binding of GR113808 and ML10302 [8]. Thus, in contrast to the β_2-adrenergic receptor, $Cys^{5.42}$ in the 5-HT_4R does not seem an additional site for ligand binding. It has also been shown for the β_2-adrenergic receptor that the $Asn^{6.55}$Leu point mutation, in TM 6, produces a substantial loss of stereospecificity for isoproterenol [11]. The β-OH-group of the ligand, which defines the chiral center, was proposed to hydrogen bond $Asn^{6.55}$. Substitution of the analogous $Asn^{6.55}$ in the 5-HT_4R by Leu abolishes the binding of serotonin [8]. However, the influence of this mutation in the binding of the GR113808 antagonist is not clear. Despite the single $Asn^{6.55}$Leu or $Phe^{6.52}$Val mutation moderately reduces the affinity for GR113808, the double $Phe^{6.52}$Ala/$Asn^{6.55}$Leu

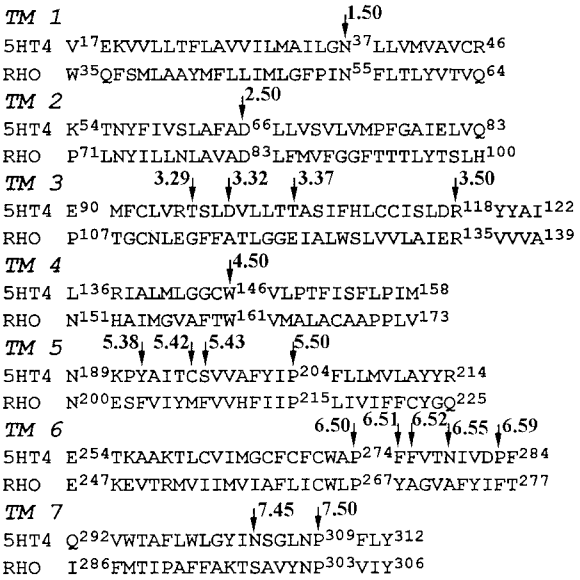


Figure 1. Alignment of the transmembrane sequences from bovine RHO and human 5-HT_4R. Numbers at the top define the general numbering scheme to identify residues in the transmembrane segments of different receptors [21]. Superscript numbers give the corresponding positions of the amino acids in the sequences of the receptor proteins.

mutation totally avoids the binding of GR113808 to the 5-HT_4R. TM 6 possesses the $Pro^{6.50}$PhePhe motif in both the adrenergic and serotoninergic subfamilies of receptors. The role of these conserved aromatic residues in ligand binding appears to be depending on the receptor family. $Phe^{6.52}$ stabilizes the interaction of the aromatic catechol-containing ring with the β_2-adrenergic receptor [12] and the interaction with certain 5-HT_{2A}R ligands [13-15]. Substitution of the adjacent $Phe^{6.51}$ has minimal effects on ligand binding in these receptors. In contrast, $Phe^{6.51}$, and not $Phe^{6.52}$, is a key residue involved in the interaction of the aromatic catechol ring with the α_{1B}-adrenergic receptor [16]. The role of these aromatic residues in the 5-HT_4R have been studied throughout the $Phe^{6.51}$Ala and $Phe^{6.52}$Leu mutations [8]. Replacement of $Phe^{6.51}$ by Ala abolishes the binding of the GR113808 antagonist, suggesting a direct interaction. $Phe^{6.52}$ substituion does not have a significant effect in either serotonin or GR113808. It is only the double $Phe^{6.52}$Ala/$Asn^{6.55}$Leu mutation (see above) that totally avoids the binding of GR113808 to the receptor. Thus, the role of $Phe^{6.52}$ and $Asn^{6.55}$ in the binding of the GR113808 antagonist remains unclear.

The structural interpretation of these experiments, investigating the structure-function relationships of GPCRs, were accomplished with molecular models of the complex between the ligands and the transmembrane domain of the receptor [8, 10, 11]. These 3-D models were derived from the high-resolution structure of bacteriorhodopsin [17] or the low-resolution structure of rhodopsin (RHO) [18, 19]. Recently, the 3-D structure of RHO was determined at 2.8 Å resolution [20]. It provides a detailed view of a GPCR in the inactive conformation of the receptor. In this work we aim to model the complex between the GR113808 antagonist and the transmembrane domain of the 5-HT_4R derived from the recent crystal structure of RHO [20]. This structure represents an appropriate template to model the 3-D structure of the 5-HT_4R because of the large number of conserved sequence patterns in the transmembrane segments [21, 22]. This computational model must offer additional structural indications about the experimentally determined role of $Asp^{3.32}$, $Ser^{5.43}$, $Phe^{6.51}$, $Phe^{6.52}$, and $Asn^{6.55}$ in the binding of GR113808 [8]. Because these residues are also present in the other members of the neurotransmitter family of 7-TM receptors, these findings will also serve for our understanding of the binding of related ligands to their cognate receptors. Moreover, the model will provide the tools for predicting the affinity of related compounds, and for guiding the design and synthesis of new ligands with predetermined affinities and selectivity.

Methods

Residue numbering scheme

We use a general numbering scheme to identify residues in the transmembrane segments of different receptors [21]. Each residue is numbered according to the helix (1 through 7) in which it is located and to the position relative to the most conserved residue in that helix, arbitrarily assigned to 50 (see Figure 1). For instance, the most conserved residue in helix 3 is designated with the index number 3.50 ($Arg^{3.50}$), the Asp preceeding the Arg is designated $Asp^{3.49}$, and the Tyr following the Arg is designated $Tyr^{3.51}$.

Molecular modeling of the transmembrane region of the 5-HT_4 receptor

The 3-D model of the transmembrane domain of the 5-HT_4R was constructed by computer-aided model building techniques from the transmembrane domain (HELIX annotation in the 1F88 PDB file) of the crystal

structure of RHO [20]. Figure 1 shows the alignment of bovine RHO and human 5-HT$_4$R (Genbank accession number Q13639) transmembrane sequences. All ionizable residues in the helices were considered uncharged with the exception of Asp$^{2.50}$, Asp$^{3.32}$, Asp$^{3.49}$, Arg$^{3.50}$ and Glu$^{6.30}$. SCWRL-2.1 was employed to add the side chains of the non-conserved residues based on a backbone-dependent rotamer library [23]. It is important to note that Thr$^{3.37}$ adopts the *gauche*-conformation. This is the only allowed conformation of Thr$^{3.37}$ due to the steric clash between the methyl group and the carbonyl oxygen of residue i-3 in the *trans* conformation [24] and the steric clash between the methyl group and the C_α (interatomic distance between heavy atoms of 2.8 Å) or C_β (3.2 Å) atoms of Pro$^{4.53}$ in the *gauche*+ conformation. Ser and Thr residues in this *gauche*− conformation induces a small bending angle in transmembrane helices because of the additional hydrogen bond formed between the O_γ atom of Ser and Thr and the i-3 or i-4 peptide carbonyl oxygen [25]. It has recently been shown that this effect is important in the 3-D conformation of the receptor [26]. Thus, a bending angle of 4° [25] has been incorporated in TM 3 at Thr$^{3.37}$. This induces the displacement of the residues located at the extracellular part of TM 3 towards TM 5, facilitating the experimentally derived interactions between the ligand and Asp$^{3.32}$ and Ser$^{5.43}$ [8].

Model of the 5-HT$_4$ receptor complexed with GR113808

The mode of recognition of GR113808 was first determined by *ab-initio* geometry optimization with the 3-21G basis set. The model system consisted on Asp$^{3.32}$, Ser$^{5.43}$ and Asn$^{6.55}$ (only the $C_\alpha$atom of the backbone is included) of the 5-HT$_4$R and the ligand GR113808 (the sulphonamide side chain attached to the piperidine nitrogen were replaced by a methyl group). All free valences were capped with hydrogen atoms. The C_α atoms of the residues were kept fixed at the positions previously obtained (see above). These optimized reduce model was used to position GR113808 inside the previously equilibrated transmembrane domain of the 5-HT$_4$R. Subsequently, the complete system was energy minimized (5000 steps). Similar procedure has been used in our recent 3-D model of the 5-HT$_{1A}$ receptor [27]. The interaction between the side chain of Asp$^{3.32}$ and the protonated piperidine ring with the side chain of Phe$^{6.51}$ was also modeled by full geometry optimization at the MP2 level of theory with the 6-31G* basis set. This procedure is capable, in principle, of describing C-H$\cdots\pi$ bonds [28].

Models of the mutant 5-HT$_4$ receptor complexed with GR113808

The helix bundles of the Asp$^{3.32}$Asn, Phe$^{6.52}$Val, Asn$^{6.55}$Leu, and Phe$^{6.52}$Ala/Asn$^{6.55}$Leu mutant receptors bound with GR113808 were constructed from the structure of the 5-HT$_4$R$\cdots$GR113808 complex, and changing the atoms implicated in the aminoacid substitutions and the conformation of GR113808 by interactive computer graphics. Subsequently, the complete systems were energy minimized (5000 steps). The interaction between Asn$^{3.32}$, in the Asp$^{3.32}$Asn mutant, and the protonated piperidine ring with Phe$^{6.51}$ was modeled by full geometry optimization at the MP2 level of theory with the 6-31G* basis set.

Quantum mechanical calculations were performed with the GAUSSIAN-98 system of programs [29]. Energy minimizations and molecular dynamics simulations were run with the Sander module of AMBER5 [30], the all-atom force field [31], SHAKE bond constraints in all bonds, a 2 fs integration time step, and a 13 Å cutoff for non-bonded interactions. Parameters for GR113808 were adapted from Cornell et al. [31] force field using RESP point charges [32].

Results and discussion

Model of the 5-HT$_4$ receptor complexed with GR113808

To identify the arragement in space of the essential determinants for recognition of the GR113808 ligand, we performed *ab-initio* geometry optimization of the ligand inside the side chains of Asp$^{3.32}$, Ser$^{5.43}$ and Asn$^{6.55}$, experimentally determined to form the ligand binding pocket [8]. Figure 2a depicts the energy-optimized structure. The complex is formed through (i) the ionic interaction between the N-H group of the protonated piperidine and the O_δ atom of Asp$^{3.32}$ at the optimized distance between heteroatoms of 2.65 Å; (ii) the hydrogen bond between the carbonylic oxygen of the ligand and the hydroxyl group of Ser$^{5.43}$ (3.00 Å); and (iii) hydrogen bond between the ether oxygen of the ligand and the amide group of Asn$^{6.55}$ (2.99 Å).

This optimized reduced model was used to position the ligand inside the transmembrane domain of

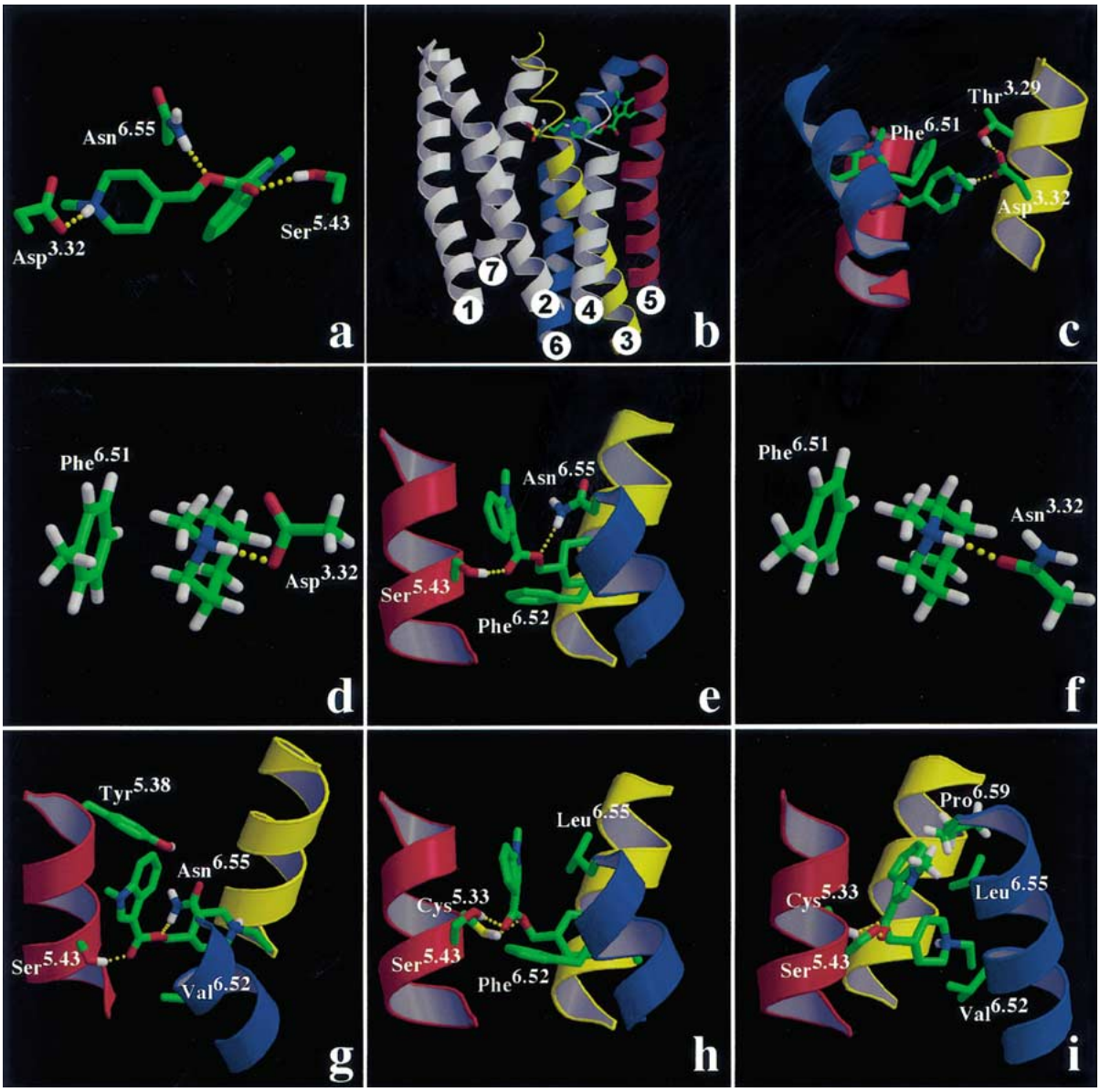


Figure 2. (a) *Ab-initio* geometry optimization, at the HF/3-21G level of theory, of GR113808 inside the side chains of $Asp^{3.32}$, $Ser^{5.43}$ and $Asn^{6.55}$. Only polar hydrogens are depicted to offer a better view. (b) Molecular model of the complex between GR113808 and the transmembrane helix bundle of the human 5-HT_4R constructed from the crystal structure of bovine RHO [20], in a view parallel to the membrane. (c, e, g, h, i) Detailed view of the transmembrane helix bundle of the 5-HT_4R (c, e) and the $Phe^{6.52}$Val (g), $Asn^{6.55}$Leu (h), and $Phe^{6.52}$Val/$Asn^{6.55}$Leu (i) mutant receptors complexed with GR113808 (the sulphonamide side chain is not depicted for clarity). The C_α traces of the extracellular part (top) of TM 3 (yellow), 5 (red), and 6 (blue) are shown. The protonated piperidine of the ligand forms an ionic interaction with $Asp^{3.32}$ and a C-H$\cdots\pi$ hydrogen bond with $Phe^{6.51}$ (c). The side chain of $Asp^{3.32}$ also forms a hydrogen bond with the neighboring side chain of $Thr^{3.29}$ (c). The carbonylic oxygen, the ether oxygen and the indole ring of the ligand hydrogen bond $Ser^{5.43}$, $Asn^{6.55}$ and $Phe^{6.52}$ (aromatic-aromatic interaction), respectively (e). The indole ring of the ligand replaces the interaction with $Phe^{6.52}$ by a similarly intense interaction with $Tyr^{5.38}$ in the $Phe^{6.52}$Val mutant (g). The mutation of $Asn^{6.55}$ to Leu replaces the hydrogen bond of the ether oxygen of the ligand from $Asn^{6.55}$ to $Cys^{5.42}$ (h). The change of orientation of the indole ring caused by the double $Phe^{6.52}$Val/$Asn^{6.55}$Leu mutant makes its N-methyl group to crash with $Pro^{6.59}$ (i). (d, f) Ab-initio geometry optimization, at the MP2/6-31G* level of theory, of $Asp^{3.32}$ in wild type receptor (d) or $Asn^{3.32}$ in the $Asp^{3.32}$Asn mutant receptor (f), the piperidine moiety of the ligand, and $Phe^{6.51}$.

the 5-HT_4R (see Figure 2b and methods for computational details). The protonated piperidine of the ligand is located between (i) $Asp^{3.32}$ and (iv) $Phe^{6.51}$ (see Figure 2c). The electron-rich clouds of the aromatic ring of $Phe^{6.51}$ interact with the electron-poor hydrogens of the carbon atoms adjacent to the protonated piperidine nitrogen of the ligand. Probably, the -CH_2- group in the side chain attached to the piperidine nitrogen achieves the larger interaction with $Phe^{6.51}$ (see Figure 2c). This type of C-H$\cdots\pi$ interaction plays a significant role in stabilizing local 3-D structures of proteins [33]. This mode of binding explains why substitution of $Phe^{6.51}$ by Ala abolishes the binding of GR113808 to the 5-HT_4R [8]. To evaluate the magnitude of this C-H$\cdots\pi$ interaction a model complex formed by the side chain of $Asp^{3.32}$, the protonated piperidine ring of the ligand, and the side chain of $Phe^{6.51}$ was optimized at the MP2 level of theory with the 6-31G* basis set (see methods for computational details). Despite the system was fully optimized with no constraints, the relative orientation of $Asp^{3.32}$, the piperidine ring of the ligand, and $Phe^{6.51}$ resembles the model of the ligand inside the 5-HT_4R (see Figures 2c and 2d). The energy of interaction (see methods for computational details) defined as the difference in energy between the optimized complex and the sum of the energies of the $Asp^{3.32}$/piperidine moieties and $Phe^{6.51}$ side chain, calculated in the conformation obtained in the complex, is -6.9 kcal/mol. Thus, there is a significant interaction of the aromatic side chain of $Phe^{6.51}$ with the $Asp^{3.32}$/piperidine fragment, despite the presence of the negatively charged Asp side chain. The importance of this C-H$\cdots\pi$ interaction is also reflected in the crystal structure of the enzyme acetylcholinesterase [34, 35], that catalyzes the hydrolysis of the neurotransmitter acetylcholine into choline. The complexes with different inhibitors, that contain trimethyl substituted amine nitrogen, revealed that the cationic side chain interact primarily with aromatic residues and not with negatively charged residues also present in the active site. Moreover, the side chain of $Asp^{3.32}$ also forms a hydrogen bond with the neighboring side chain of $Thr^{3.29}$ (Figure 2c).

The indole ring of GR113808 is pointing towards (v) $Phe^{6.52}$ (see Figure 2e). The Phe side chain is positioned in the face-to-edge orientation (T-shaped) to the indole ring. This type of π-σ aromatic-aromatic interaction has been described as a protein structure stabilization [36]. $Phe^{6.52}$ act as hydrogen bond acceptor (π electrons) and the indole ring of GR113808 act as hydrogen bond donor (the C-H bond). Finally, the sulphonamide side chain attached to the piperidine nitrogen is pointing towards TM 7 and interacting with $Asn^{7.45}$ (results not shown).

Model of the $Asp^{3.32}$Asn mutant receptor

In the $Asp^{3.32}$Asn mutant receptor the N-H group of the protonated piperidine moiety of the ligand (i) forms a charged hydrogen bond with the O_δ atom of $Asn^{3.32}$ (see Figure 2f) replacing the ionic interaction with the O_δ atom of $Asp^{3.32}$ in the wild type receptor (see above). However, this significant modification of the mode of binding of the ligand does not decrease the affinity of GR113808 for the receptor [8].

The affinity constant is function of the interaction energy between the ligand and the receptor, the energy required to displace the ligand from the extracellular aqueous environment to the binding pocket of the receptor, and the energy required to change the conformation of the receptor from the unbound to the bound state. Thus, the following factors must be taken into account. First, the $Asp^{3.32}$Asn mutation decreases the binding of the N-H moiety of the protonated piperidine, from an ion pair ($Asp^{3.32}$) to a charged hydrogen bond ($Asn^{3.32}$). However, substitution of a negatively charged side chain (Asp) by a neutral side chain (Asn) increases the explicit charge in the hydrogens of the carbon atoms adjacent to the protonated piperidine nitrogen and accordingly the interaction with $Phe^{6.51}$. Thus, in order to estimate this effect we evaluated the interaction between $Phe^{6.51}$ and the protonated piperidine ring of the ligand hydrogen bonded to $Asn^{3.32}$, in a similar manner to the interaction of $Phe^{6.51}$ with the $Asp^{3.32}$/piperidine fragment in the wild type receptor (see above and methods). The fully optimized complex is depicted in Figure 2f. The calculated interaction energies confirm the previous hypothesis that the interaction of $Phe^{6.51}$ with the $Asn^{3.32}$/piperidine fragment (-11.0 kcal/mol) is stronger than with the $Asp^{3.32}$/piperidine fragment (-6.9 kcal/mol), partially compensating the decrease of binding energy due to the substitution of $Asp^{3.32}$ by Asn. The absence in serotonin of the piperidine ring or more importantly the $-CH_2$ group in the side chain attached to the piperidine nitrogen explains why the $Asp^{3.32}$Asn mutation has a significant effect in the binding of serotonin [8]. Notably, both ML10302 and ML10375 ligands possess the piperidine ring and the effect of the $Asp^{3.32}$Asn mutation is only partial. The magnitude of the $Asp^{3.32}$Asn mutation in piperidine-containing ligands will depend in the mode of binding

and the relative orientation of Phe$^{6.51}$ to stabilize the complex through the proposed C-H$\cdots\pi$ interaction.

Second, in the absence of the ligand the side chain at position 3.32 (Asp$^{3.32}$ in wild type or Asn$^{3.32}$ in the mutant receptor) is coordinated with other side chains of the receptor. For example, it has been shown for the α_{1B}-adrenergic receptor that Asp$^{3.32}$ interacts in the ligand-free form with a Lys residue in TM 7 [37, 38]. Thus, the process of ligand binding requires the partial or total disruption of the side chain environment at position 3.32. Clearly, this side chain reorganization will require a larger energetical cost for Asp than for Asn.

This data suggests that GR113808 possess similar affinity for the Asp$^{3.32}$Asn mutant receptor than for wild type receptor [8], because the loss of binding affinity from an ion pair (Asp) to a charged hydrogen bond (Asn) is compensated by the larger energetical penalty of Asp to disrupt its side chain environment, and the larger interaction between Phe$^{6.51}$ and the piperidine ring of the ligand in the Asp$^{3.32}$Asn mutant.

Model of the Phe$^{6.52}$Val mutant receptor

In the Phe$^{6.52}$Val mutant the indole ring of GR113808 modifies the conformation observed in the binding mode to wild type receptor (see Figure 2e) and replaces the π-σ aromatic-aromatic interaction with (v) Phe$^{6.52}$ by a similarly intense π-σ interaction with Tyr$^{5.38}$ (see Figure 2g). The Tyr side chain is also positioned in the face-to-edge orientation to the indole ring. In this mode of binding the indole ring of GR113808 act as hydrogen bond acceptor (π electrons) and Tyr$^{5.38}$ act as hydrogen bond donor (the C-H bond). Phe$^{6.52}$Val substitution does not have a significant effect in the binding of GR113808 [8], because the Phe$\cdots$indole interaction is similar in magnitude to the indole$\cdots$Tyr interaction.

Model of the Asn$^{6.55}$Leu mutant receptor

Substitution of Asn$^{6.55}$ by Leu does not allow the ether oxygen of GR113808 to hydrogen bond (iii) the amide group of Asn$^{6.55}$ as in wild type receptor (see above). Thus, the absence of Asn$^{6.55}$ drives the ether oxygen of the ligand to hydrogen bond Cys$^{5.42}$ while the carbonylic oxygen remains hydrogen bonded to Ser$^{5.43}$ (see Figure 2h). This hydrogen bond network resembles the suggested binding of the meta-hydroxyl group of catecholamine ligands to Ser$^{5.42}$ and Ser$^{5.43}$ in the β_2-adrenergic receptor [10]. It is important to note that the indole ring of GR113808 is pointing towards the intracellular side to achieve the π-σ aromatic-aromatic interaction with (v) Phe$^{6.52}$ as in wild type receptor (see above). This mode of binding orients the N-methyl substituent of the indole ring towards the extracellular side (see Figure 2h).

Clearly, the $-$SH moiety of Cys$^{5.42}$ cannot hydrogen bond the ether oxygen of the ligand with the same strength as the $-NH_2$ moiety of Asn$^{6.55}$. Thus, the Asn$^{6.55}$Leu mutation reduces the affinity of GR113808 by a factor of 5.2 [8] that represents a free energy change of 1.0 kcal/mol. This approximately equals the free energy difference between the SH$\cdots$O and NH$\cdots$O hydrogen bonds.

In contrast, the Asn$^{6.55}$ to Leu substitution abolishes the binding of serotonin [8]. Thus, the mode of binding of serotonin to Asn$^{6.55}$ differs from GR113808. The absence of the second hydrogen bond acceptor group in serotonin (the ether oxygen in GR113808) makes the indole ring of serotonin to hydrogen bond Asn$^{6.55}$ (results not shown). The hydrogen bond is formed between the π electron-rich clouds of the aromatic ring and the electron-poor $-NH_2$ hydrogens of Asn$^{6.55}$ in a similar manner to the proposed hydrogen bond between benzene and water [39]. Clearly, in the Asn$^{6.55}$Leu mutant receptor the indole ring of serotonin cannot hydrogen bond Cys$^{5.42}$ as the ether oxygen of GR113808 does with the observed loss of binding affinity.

Model of the Phe$^{6.52}$Val/Asn$^{6.55}$Leu double mutant receptor

Surprisingly, the double Phe$^{6.52}$Val/Asn$^{6.55}$Leu mutant avoids the binding of GR113808, despite the single Phe$^{6.52}$Val or Asn$^{6.55}$Leu mutations have none or small effect [8]. In this double mutant receptor the absence of Asn$^{6.55}$ forces the ether/carbonylic oxygens of the ligand to hydrogen bond Cys$^{5.42}$ and Ser$^{5.43}$, as in the Asn$^{6.55}$Leu mutant receptor (see above); and the absence of Phe$^{6.52}$ forces the indole ring of the ligand to point towards the extracellular, as in the Phe$^{6.52}$Val mutant (see above). However, in the Phe$^{6.52}$Val single mutant receptor the hydrogen bond to Ser$^{5.43}$ and Asn$^{6.55}$ places the indole ring between TM 5 and 6 (see Figure 2g), whereas in the Phe$^{6.52}$Val/Asn$^{6.55}$Leu double mutant the hydrogen bond to Cys$^{5.42}$ and Ser$^{5.43}$ places the indole ring towards TM 6 (see Figure 2i). This small change in the orientation of the indole ring makes its N-methyl group to crash with Pro$^{6.59}$ in TM 6 (see Figure 2i). This mode of binding explains the lack of affinity

of GR113808 for the double Phe$^{6.52}$Val/Asn$^{6.55}$Leu mutant receptor [8].

Conclusions

The recognition of the GR113808 antagonist by the transmembrane domain of the human 5-HT$_4$R consist of: (i) the ionic interaction between the N-H group of the protonated piperidine of the ligand and Asp$^{3.32}$; (ii) the hydrogen bond between the carbonylic oxygen of the ligand and Ser$^{5.43}$; (iii) the hydrogen bond between the ether oxygen of the ligand and Asn$^{6.55}$; (iv) the hydrogen bond between the C-H groups adjacent to the protonated piperidine nitrogen and the π electrons of Phe$^{6.51}$; and (v) the π-σ aromatic-aromatic interaction between the indole ring of the ligand and Phe$^{6.52}$. This derived computational model has provided additional structural interpretation of the mutagenesis experiments aimed to test the role of Asp$^{3.32}$, Ser$^{5.43}$, Phe$^{6.51}$, Phe$^{6.52}$, and Asn$^{6.55}$ on the binding affinity of GR113808 to the 5-HT$_4$R [8]. This recognition model, together with the postulated pharmacophore model for the binding of 5-HT$_4$R antagonists [40], will be used to guide the design of new antagonists with predetermined affinities and selectivity. These studies are now in progress and the results will be reported in due course.

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