The Serotonin Binding Site of Human and Murine 5-HT\textsubscript{2B} Receptors

MOLECULAR MODELING AND SITE-DIRECTED MUTAGENESIS

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Bacteriorhodopsin and rhodopsin crystal structures were used as templates to build structural models of the mouse and human serotonin (5-HT)-2B receptors (5-HT\textsubscript{2BR}). Serotonin was docked to the receptors, and the amino acids predicted to participate to its binding were subjected to mutagenesis. 5-HT binding affinity and amino acids predicted to participate to its binding were measured in LMTK+ cells transfected with either wild-type or mutated receptor genes. According to these measurements, the bacteriorhodopsin-based models of the 5-HT\textsubscript{2BR} appear more confident than the rhodopsin-based ones. Residues belonging to the transmembrane domains 3 and 6, \textit{i.e.} Asp\textsubscript{3.32}, Ser\textsubscript{3.36}, Phe\textsubscript{6.52}, and Asn\textsubscript{6.55}, make direct contacts with 5-HT. In addition, Trp\textsubscript{3.28}, Phe\textsubscript{3.35}, Phe\textsubscript{6.52}, and Phe\textsubscript{7.38} form an aromatic box surrounding 5-HT. The specificity of human and mouse 5-HT\textsubscript{2BR} may be reflected by different rearrangements of the aromatic network upon 5-HT binding. Two amino acids close to Pro\textsuperscript{5.50} in the human transmembrane domain 5 sequence were permuted to introduce a “mouse-like” sequence. This change was enough to confer the human 5-HT\textsubscript{2BR} properties similar to those of the mouse. Taken together, the computed models and the site-directed mutagenesis experiments give a structural explanation to (i) the different 5-HT \textit{pK}\textsubscript{D} values measured with the human and mouse 5-HT\textsubscript{2BR} (7.9 and 5.8, respectively) and (ii) the specificity of 5-HT binding to 5-HT\textsubscript{2BR} as compared with other serotonergic G-protein coupled receptors.

Of the 14 mammalian serotonin (5-hydroxytryptamine (5-HT)) receptor subtypes, all but one (5-HT\textsubscript{\textasteriskcentered}) belong to the super-family of G-protein-coupled receptors (GPCRs) (1). The 5-HT\textsubscript{\textasteriskcentered} subtype comprises three closely related receptors, which are 5-HT\textsubscript{2A}, 5-HT\textsubscript{2B}, and 5-HT\textsubscript{2C}. Amino acid sequences of the rat (2), mouse (3), and human (4) 5-HT\textsubscript{2B} receptors (5-HT\textsubscript{2BR}s) are highly similar at the level of the predicted transmembrane domains (TMDs); they exhibit 88, 82, and 79% homology upon comparison of mouse and rat, human and mouse, and human and rat receptors, respectively (5). Moreover, significant correlations were established between the pharmacological profiles of human and rat (or human and mouse) 5-HT\textsubscript{2BR} but not between those of mouse and rat (5). Nevertheless, some compounds (\textit{e.g.} certain ergolines and benzoylpiperidines) can be used to discriminate pharmacologically between human and rat 5-HT\textsubscript{2BR} (6).

Structural descriptions of these receptors and of their binding interactions are required to rationalize the above findings. In the absence of any 5-HT receptor three-dimensional structure, computer modeling studies were undertaken in the present study. Our aim was to explain the different 5-HT \textit{pK}\textsubscript{D} values measured for the rat, mouse, and human 5-HT\textsubscript{2BR} (7.5, 5.8, and 7.9, respectively) and to better understand the mechanism of 5-HT\textsubscript{2BR} activation.

Two main strategies have already been used to build GPCR three-dimensional models. The first one relied on the use of the structure of bacteriorhodopsin (BR) at a 3-Å resolution as a homology template (7, 8). The second strategy, already used for three-dimensional-modeling of the 5-HT\textsubscript{2A} (9, 10) and 5-HT\textsubscript{2C} receptors (10, 11), aimed at constructing models \textit{de novo} by only using the 9-Å structure of bovine rhodopsin (RH) as a guide to orient the TMDs. These studies were the starting point in the elucidation of the conformational switch between the inactive and active states of GPCRs. A key feature of these approaches involved the replacement of the network of constraints in the inactive ground state of the receptor by a ligand-induced set of interactions in the activated state (12–14).

In the present work, the construction of three-dimensional models of the mouse and human 5-HT\textsubscript{2BR} was carried out using either the high resolution BR structure or the recently described crystal structure of bovine RH (15, 16) as templates. Structural models of the native receptors liganded or not with 5-HT could be derived. In a second step, site-directed mutagenesis experiments were performed to validate the models. Residues predicted to participate to 5-HT binding in either the human or the mouse 5HT\textsubscript{2BR} were changed. 5-HT binding affinity and 5-HT-induced inositol triphosphate (IP\textsubscript{3}) production were systematically assessed with each mutated receptor. As a result, the involvement of 5-HT binding of several amino acid residues could be established. Moreover, a two-amino acid permutation in the TMD5 of the human receptor was performed to introduce a mouse-like sequence. This modification

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‡ The abbreviations used are: 5-HT, serotonin; 5-HT\textsubscript{2BR}, serotonin-2B receptor; BR, bacteriorhodopsin; GPCR, G-protein-coupled receptor; IP\textsubscript{3}, inositol trisphosphate; MD, molecular dynamics; RH, rhodopsin; r.m.s.d., root mean square deviation; TMD, trans-membrane domain; ALF, fluorescent sequencing system.

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were aligned with BR and RH using the CLUSTAL X software (22). For clarity, only the TMD sequences of the two templates and of the two studied 5-HT<sub>2BR</sub>Rs are presented in Fig. 1.

Model refinements, energy minimization, and molecular dynamics (MD) simulations were performed with the CHARMM program (23). A 20-ps molecular-mechanics simulation was applied to the minimized protein models used in the MD simulations. Each receptor model was energy-minimized for 100 ps and then equilibrated for 800 ps. The equilibrated structures of ligand/receptor complexes were obtained by averaging the energy-minimized structures over the third simulation step. Ligand docking to the receptor models was performed with the Dock program (24). The corresponding relative free energies are in satisfactory agreement with those of the conformers obtained from semi-empirical (Intermediate Neglect Differential Overlap and Perturbative Configuration Interaction using Localized Orbitals) calculations (26).

Docking of 5-HT to the 40 obtained receptor models was performed in the vicinity of the negatively charged residues located at the inward side of the receptors (Asp<sub>7.32</sub>, Glu<sub>7.36</sub>, Asp<sub>7.38</sub>), referring to the most conserved BR and RH-specific sequences used as structural templates are shown in green and purple, respectively. Amino acid residues subjected to site-directed mutagenesis are shown in red.

**EXPERIMENTAL PROCEDURES**

**Molecular Modeling—**Three-dimensional-models of the TMD bundle of the human and mouse 5-HT<sub>2BR</sub>Rs were built using criteria and procedures that took into account sequence conservation (17, 18) and of the two studied 5-HT<sub>2BR</sub>Rs are presented in Fig. 1. Amino acid sequence alignments of BR, RH, and mouse and human 5-HT<sub>2BR</sub>TMDs. Each helix amino terminus starting residue is numbered according to SWISSPROT data bank sequences (BR, P02945; mouse 5-HT<sub>2BR</sub> receptor, Q02152; human 5-HT<sub>2BR</sub> receptor, P41595; RH, P20699). In addition, a consensus numbering scheme (Ref. 20; see “Experimental Procedures”), referring to the most conserved residue (shown in yellow and shaded in gray) of each TMD among the GPCR superfamily, was used. BR- and RH-specific sequences used as structural templates are shown in green and purple, respectively. Amino acid residues subjected to site-directed mutagenesis are shown in red.

resulted in a lowering of the 5-HT affinity and in a change of the IP<sub>3</sub> production to values similar to those measured with the mouse 5-HT<sub>2BR</sub>R. We conclude that the TMD5 region encompassing the mutated residues participates in the species signature of the 5-HT<sub>2BR</sub>Rs.

**Site-directed Mutagenesis—**Full coding regions of the human or mouse 5-HT<sub>2BR</sub>R cDNAs (3, 4) were subcloned in the mammalian expression vector pRC/CMV (Invitrogen). Mutations were introduced using the QuikChange site-directed mutagenesis kit (Stratagene) and verified by DNA sequencing with an automated fluorescent sequencing system (ALF, Amersham Biosciences).

**Ligand Binding and Coupling Assays—**Pharmacological and functional properties of the engineered receptors, wild-type or mutated cDNAs were stably transfected in LMTK<sup>−</sup> cells (27). To avoid transcriptional bias at the level of the expression of the 5-HT<sub>2BR</sub>R variants, cell lines expressing comparable amounts of RNA from cDNA were selected by Taqman<sup>®</sup> real-time PCR. Equilibrated structures of ligand-receptor complexes were obtained by averaging the energy-minimized structures over the third simulation period. Point mutations were introduced in the models of the mouse and human 5-HT<sub>2BR</sub>Rs by replacing the side chain of a wild-type residue. The positioning of each mutated residue was optimized as for the wild-type residues. All atoms of the receptor were held fixed except those of the mutated residue, and a short minimization step was applied. The receptor atom coordinates were then allowed to relax. Ligand docking to the variant receptors and simulations of the ligand/receptor complexes were performed as above. Conformational stability likelihood of the generated 5-HT<sub>2BR</sub>R models were systematically assessed by following the root mean square deviations of the cartesian coordinates of backbone atoms at all steps of the simulations. Point mutations were introduced in the models of the mouse and human 5-HT<sub>2BR</sub>Rs by replacing the side chain of a wild-type residue. The positioning of each mutated residue was optimized as for the wild-type residues. All atoms of the receptor were held fixed except those of the mutated residue, and a short minimization step was applied. The receptor atom coordinates were then allowed to relax. Ligand docking to the variant receptors and simulations of the ligand/receptor complexes were performed as above. Conformational stability likelihood of the generated 5-HT<sub>2BR</sub>R models were systematically assessed by following the root mean square deviations of the cartesian coordinates of backbone atoms at all steps of the simulations. Point mutations were introduced in the models of the mouse and human 5-HT<sub>2BR</sub>Rs by replacing the side chain of a wild-type residue. The positioning of each mutated residue was optimized as for the wild-type residues. All atoms of the receptor were held fixed except those of the mutated residue, and a short minimization step was applied. The receptor atom coordinates were then allowed to relax. Ligand docking to the variant receptors and simulations of the ligand/receptor complexes were performed as above. Conformational stability likelihood of the generated 5-HT<sub>2BR</sub>R models were systematically assessed by following the root mean square deviations of the cartesian coordinates of backbone atoms at all steps of the simulations.
Addition of 100 μl of fetal calf serum-free Dulbecco's modified Eagle's medium supplemented with 2 μl [3H]-5-HT and various concentrations of unlabeled 5-HT. After a 15-min incubation, cells were washed twice with cold Dulbecco's modified Eagle's medium and 2 ml of 1 N HClO4 were added. Radioactivity was counted in a 500-μl fraction using a liquid scintillation counter (Packard Instrument Co.). The specific binding (mean 38%) corresponded to the difference observed in the absence and presence of 10 μM unlabeled 5-HT. The 5-HT-induced IP3 amounts produced by the cells were measured as described previously (27). Data were analyzed using the iterative non-linear regression fitting program Ligand (version 3.0) and RS/1 (release 4.0).

RESULTS

Computer Modeling of Human and Mouse Wild-type 5-HT2B Receptors—As a first step, the seven TMDs of the 5-HT2BR models were organized in a counterclockwise arrangement. In the case of the RH-based modeling, constraints resulting from the alignment of the 5-HT receptor sequences to the template led to an electrostatic interaction between Asp2.50(h100,m99) and Asp2.32(h135,m134). This interaction was already evidenced between Asp50(120) and Asn49(396) in the human 5HT2AR (28). With the BR-based models, the orientation of TMD7 with respect to TMD2 had to be slightly modified to establish a similar interaction between Asp50(120) and Asn49(396) in the human 5HT2AR (28).

By introducing this constraint in the BR-based models, 8 of the 10 starting low energy structures could be retained (see “Experimental Procedures”). However, in these structures, Glu3.36(h363,m362) pointed the phospholipid bilayer instead of the inward face of the receptor. We thus rotated TMD7 to point Glu7.36(h363,m362) toward the central core of the receptors. Such a rotation, which slightly moved Asn6.55(h344,m343) away from Asp3.32(h135,m134), allowed Glu7.36(h363,m362) to interact directly with 5-HT in the BR-based models (see below). Asp3.32(h135,m134) also interacted with Asn6.55(h344,m343) via a hydrogen bond network in both the BR- and RH-based models of 5-HT2BRs. The resulting H-bond network conferred stability to the packing of TMDs 1, 2, and 7.

Docking of 5-HT to Human and Mouse Wild-type 5-HT2B Receptors—In the presence of 5-HT, significant conformational changes occurred during the first 50 ps of the 310 K MD simulations. Relaxed structures were obtained after 100 ps. The low r.m.s.d. values obtained during the preparative 10 K MD phase are likely to reflect the low temperature used. Similarly to what was observed with the unliganded receptors, motions of TMDs 5 and 6 and, to a lesser extent, TMD7, mainly contributed to the observed r.m.s.d. in the liganded receptors (Table I).

The r.m.s.d. of the backbone atoms of each form of the 5-HT2BR models was significantly lower for the RH-based models compared to the BR-based models (Table I). This difference is likely to result from stronger hydrogen bonding between the indolic NH group of 5-HT and the carboxylate group of Asp3.32(h135,m134) and Asp6.55(h344,m343). These features could also be observed in the RH-based models. However, in the RH-based models, the carboxylate group of Asp6.55(h344,m343) no longer interacted with the cationic amino group of 5-HT. Rather, it interacted weakly with the 5-HT indolic NH group. During the simulations, the 5-HT molecule moved toward TMDs 6 and 7. This translation was more pronounced in the case of the human 5-HT2BR than in the case of the mouse 5-HT2BR. As a consequence, the interaction between the hydroxyl group of 5-HT and the OH group of Ser3.36(h139,m138) would be weakened.

In the course of MD simulations of the RH-based models (Figs. 2, a and b, and 3, a and c), we also noticed that the 5-HT cationic amino group interacted with the carboxylate group of Asp3.32(h135,m134) and that the 5-HT hydroxyl group was hydrogen bonded by the OH group of Ser3.36(h139,m138). These features could also be observed in the RH-based models. However, in the RH-based models, the carboxylate group of Asp6.55(h344,m343) no longer interacted with the cationic amino group of 5-HT. Rather, it interacted weakly with the 5-HT indolic NH group. During the simulations, the 5-HT molecule moved toward TMDs 6 and 7. This translation was more pronounced in the case of the human 5-HT2BR than in the case of the mouse receptor. The relative TMD motions in the 5-HT2BR RH-based models were similar to those in the BR-based models. In particular, r.m.s.d. values were larger for TMD5 and -6 backbone atoms. In both BR- and RH-based models complexed with 5-HT, motions of proline residues belonging to TMDs 5 and 6 accompanied the rearrangement of the hydrophobic pocket including Trp6.52(h341,m340) and Phe6.52(h341,m340). Motions of Phe6.52(h341,m340) and Trp6.48(h337,m342) also accompanied the
5-HT translation. This motion had, however, a smaller amplitude with the mouse 5-HT2BR than with the human. After completion of the MD simulations with the eight low energy structures previously selected, (i) the 5-HT cationic group still interacted with Asp3.32(h135,m134), (ii) the 5-HT hydroxyl group was H-bonded to the hydroxyl group of Ser3.36(h139,m138), and (iii) new van der Waals contacts formed between 5-HT and Val3.33(h136,m135) in the case of both receptors, between 5-HT and Leu3.29(m131) with mouse, and between 5-HT and Trp3.28(h131) plus Val7.39(h366) with human. In the two receptors, the 5-HT indolic NH group still pointed toward the oxygen atom of Asn6.55(h344,m343). However, the resulting electrostatic bond appeared weaker with the human receptor, because of the relatively large translation of 5-HT toward TMDs 6 and 7. This difference may reflect the different sets of van der Waals contacts observed in the liganded human and mouse 5-HT2BRs.

Functional Analysis of Human and Mouse Mutant 5-HT2B Receptors—Three-dimensional modeling of the human and mouse 5-HT2BRs suggests that 5-HT binding relies on a limited number of residues. To assess the contribution of these amino acids to the species-specific pharmacology of 5-HT2BRs, mutant receptors were engineered by site-directed mutagenesis. The 5-HT binding properties (\(K_d\), \(B_{max}\)) and the 5-HT-induced efficacy (\(E_{C50}\) and \(E_{max}\) of IP3 production) were measured in stably transfected LMTK+ cells expressing variant receptors (Table II). Cells transfected with the corresponding wild-type receptor were used for comparison. To avoid transcriptional variations between cell strains, clones expressing comparable amounts of 5-HT2BR mRNA were selected by Taqman analysis and used in further biochemical and pharmacological experiments.

D135A Human and D134A Mouse 5-HT2BRs—In the 5-HT2BR (BR- or RH-based) models, Asp3.32(h135,m134) side chain acts as the primary counterion of the protonated amino group of 5-HT. The D(h135,m134)A mutation in the two 5-HT2BR sequences (human and mouse) markedly decreased both the 5-HT binding affinity and the transduction efficacy.

S139A Human 5-HT2BR—Ser3.36(h139) was predicted to be involved in 5-HT binding in both the BR- and the RH-based models. The S139A mutation affected neither the receptor expression level (\(B_{max}\)) nor the maximal 5-HT-induced IP3 production (\(E_{max}\)), whereas the binding affinity for 5-HT was reduced about 30-fold. Such a factor underlines the crucial role of Ser-139 in 5-HT binding.

N344A, S222A, and S222A/N344A Human 5-HT2BR—In either the BR- or the RH-based models of 5-HT2BRs, the 5-HT hydroxyl group was suspected to directly interact with 5-HT. In agreement with this prediction, substitution of this residue by Ala in the human receptor led to a decrease (7-fold) of the 5-HT binding affinity. Other assayed properties of the receptor remained unchanged.

Ser5.43(h222,m221), a conserved residue in the 5-HT2R family (21), is likely to be involved in the H-bonding of the 5-HT hydroxyl group. Surprisingly, the S222A mutation did not interfere either with the 5-HT binding affinity or with the IP3
production. It only induced a 2-fold decrease of the expression level of the receptor. These results bring support to the BR-based models of the human (a and b) and mouse (c and d) 5-HT$_{2B}$ receptors. 5-HT is at the center of the binding pocket of the 5-HT$_{2B}$ receptors. D3.32 (Asp$^{3.32}$h$_{135}$m$_{134}$) is the primary counter-ion that interacts with the 5-HT amino group. S3.36 (Ser$^{3.36}$h$_{139}$m$_{138}$) H binds the OH group of 5-HT. N6.55 (Asn$^{6.55}$h$_{344}$m$_{343}$) stabilizes 5-HT in its binding pocket through interactions with either the indole ring or the amino group of the neurotransmitter depending on the template used for the modeling. Note the larger 5-HT binding site in the human 5-HT$_{2B}$ receptor (a and b) than in the mouse (c and d).

It is of note that structure-function studies based on mutagenesis experiments of a single residue followed by functional analyses, such as the measurement of the affinity of a single ligand, cannot distinguish between either a modification of contacts between the receptor and the ligand or conformational changes within the overall receptor structure. Thus, because Asn$^{6.55}$h$_{344}$ might compensate for the loss of Ser$^{5.43}$h$_{222}$ in the RH-based 5-HT$_{2B}$R models, we decided to engineer the double...

FIG. 3. Stereoview of the 5-HT molecule in the binding site of RH (a and c)- and BR (b and d)-based models of human (a and b) and mouse (c and d) 5-HT$_{2B}$ receptors. 5-HT is at the center of the binding pocket of the 5-HT$_{2B}$ receptors. D3.32 (Asp$^{3.32}$h$_{135}$m$_{134}$) is the primary counter-ion that interacts with the 5-HT amino group. S3.36 (Ser$^{3.36}$h$_{139}$m$_{138}$) H binds the OH group of 5-HT. N6.55 (Asn$^{6.55}$h$_{344}$m$_{343}$) stabilizes 5-HT in its binding pocket through interactions with either the indole ring or the amino group of the neurotransmitter depending on the template used for the modeling. Note the larger 5-HT binding site in the human 5-HT$_{2B}$ receptor (a and b) than in the mouse (c and d).
Table II

5-HT binding characteristics and 5-HT-induced IP₃ production in LMTK cells stably transfected with wild-type or mutant 5-HT₂B receptors

| 5-HT₂B receptors | Kᵢ | Bₘₐₓ | E₃₅₀ | E₅₀₀ |
|------------------|----|-----|------|------|
| Wild-type Mouse  | 1,445 ± 141ᵃ | 408 ± 48 | 676 ± 41ᵃ | 761 ± 64 |
| Wild-type Human  | 12 ± 2ᵇ | 392 ± 39 | 9 ± 1ᵇ | 804 ± 58 |
| Mutant TMD1      | 1,514 ± 170ᵃ | 396 ± 28 | 794 ± 52ᵃ | 792 ± 46 |
| TMD2 Human D100N | 148 ± 15ᵃᵇ | 441 ± 38 | 120 ± 12ᵃᵇ | 364 ± 82ᵃᵇ |
| TMD3 Mouse D134A | >10,000ᵃᵇ | ND     | >10,000ᵃᵇ | ND     |
| Human D135A      | >10,000ᵃᵇ | ND     | >10,000ᵃᵇ | ND     |
| Human S139A      | 347 ± 13ᵃᵇ | 423 ± 46 | 17 ± 1ᵇ | 787 ± 88 |
| TMD4 Human A187S | 9 ± 1ᵇ | 211 ± 16ᵇ | 9 ± 1ᵇ | 806 ± 76 |
| TMD5 Human S222A | 11 ± 2ᵇ | 189 ± 19ᵇ | 11 ± 1ᵇ | 794 ± 71 |
| Human T228A      | 1,086 ± 169ᵃ | 398 ± 32 | 186 ± 12ᵃᵇ | 168 ± 37ᵇ |
| Human P229A      | 10 ± 2ᵇ | 200 ± 19ᵇ | 14 ± 1ᵇ | 814 ± 48 |
| Human A231T      | 93 ± 16ᵇ | 368 ± 32 | 56 ± 15ᵇ | 181 ± 27ᵇ |
| TMD6 Human W337A | 1,230 ± 14ᵇ | 394 ± 45 | 795 ± 51ᵃ | 152 ± 17ᵇ |
| Human F341A      | 132 ± 16ᵇ | 344 ± 26 | 13 ± 1ᵇ | 788 ± 80 |
| Human N344A      | 174 ± 13ᵇ | 422 ± 37 | 14 ± 1ᵇ | 786 ± 81 |
| TMD7 Human E363A | 83 ± 15ᵇ | 354 ± 28 | 12 ± 2ᵇ | 787 ± 82 |
| Human N376D      | 58 ± 15ᵇ | 181 ± 18ᵇ | 11 ± 1ᵇ | 796 ± 76 |
| TMDs 2 + 7: human D100N/N376D | 141 ± 16ᵇ | 342 ± 25 | 120 ± 13ᵇ | 296 ± 59ᵇ |
| TMDs 5 + 6: human S222A/N344A | 85 ± 14ᵇ | 353 ± 36 | 10 ± 1ᵇ | 798 ± 78 |

ᵃ Significant (p < 0.05) difference vs. the human wild-type receptor.
ᵇ Significant (p < 0.05) difference vs. the mouse wild-type receptor.

mutant S222A/N344A. With this mutant, the decrease in 5-HT affinity was of similar magnitude than that observed with the N344A single mutant. This result strengthens the idea that Ser⁷⁺⁴³(h²²²) has no direct role in the binding of 5-HT to the human 5-HT₂B receptor.

W337A and F341A Human 5-HT₂B receptors—The aromatic residues Trp⁶⁺³⁸(h³³³) and Phe⁶⁺⁴⁰(h³⁴⁰) were also predicted to be involved in 5-HT binding and/or IP₃ production. Mutation of any of these residues significantly decreased (11–15-fold) the binding affinity of 5-HT. In contrast, the receptor expression level and the 5-HT-induced IP₃ synthesis remained insensitive to the mutations.

D100N, N376D, and D100N/N376D Human 5-HT₂B receptors—Mutations of either Asp²⁺⁵⁰(c¹⁰⁰) or Asn⁷⁺⁴⁹(c²⁷⁶) did not affect the receptor expression. However, the 5-HT affinity was decreased by 12-fold, and the maximal 5-HT-induced IP₃ production was reduced by 2.5-fold. These effects disappeared upon construction of the double mutant receptor D100N/N376D. Such results nicely illustrate the electrostatic interaction established between these two amino acids. We may therefore conclude that whatever the template (BR or RH) used, the interaction between TMDs 2 and 7 is of crucial importance for 5-HT binding and 5-HT-induced IP₃ production.

E363A Human 5-HT₂B receptors—Only in the BR-based models of 5-HT₂B receptors, Glu⁷⁺⁴⁹(c²⁷⁶) seems to be involved in 5-HT binding. Through rotation of TMD7, Asn⁷⁺⁴⁹(c²⁷⁶) and Asp²⁺⁵⁰(c¹⁰⁰) can be allowed to interact with the side chain of the Glu⁷⁺⁴⁹(c²⁷⁶) residue, which points toward the inward face of the receptor. However, because Asn⁷⁺⁴⁹(c²⁷⁶) and Glu⁷⁺⁴⁹(c²⁷⁶) are separated by almost a helix half-turn, the interaction between Glu⁷⁺⁴⁹(c²⁷⁶) and the indolic NH group of 5-HT should be relatively weak. Indeed, the amplitude of the decreases in both the expression level (2-fold) and the 5-HT binding affinity (5-fold) of the E363A mutant remained modest.

A187S Human 5-HT₂B receptor—A serine is conserved at the 4.57 locus of all GPCRs except 5-HT₂B receptors in which an alanine is present. In a RH-based model of 5-HT₂B receptor, Ser⁷⁺⁴³(h²²²) was reported to form a hydrogen bond with the NH indolic group of 5-HT (26). According to our RH-based models of 5-HT₂B receptor, an A187S substitution should restore the H-bond interaction with this indolic group. Such a change should also reinforce the interaction of the 5-HT hydroxyl group with Ser⁷⁺⁴³(h²²²) and disrupt the contact between 5-HT and Asn⁷⁺⁴⁹(c²⁷⁶), a residue involved in the binding site. In contrast, in our BR-based models of the human 5-HT₂B-A187S mutant, the H-bond between the NH indolic group of 5-HT and the substituted Ser does not occur. Actually, no variation of either 5-HT binding affinity or the IP₃-induced efficacy could be detected with the A187S mutant if compared with the wild-type receptor. The receptor expression level only reduced the expression level by 2-fold (Table II).

A79S Mouse 5-HT₂B receptors—Among the 6 “mouse-specific” (versus human and rat 5-HT₂B receptor) sequences, TMD residues (Ala¹⁺³⁶(m⁷⁶), Ile⁷⁺⁴⁶(m⁹⁷), Val¹⁺⁵⁰(m²¹⁸), Ala¹⁺⁵⁶(m²²⁴), Val¹⁺⁵⁹(m³²⁷), and Leu⁷⁺⁵⁰(m³⁴⁴)). Fig. 1), Ala¹⁺⁵⁸ replaces a Ser residue present in all other members of the 5-HT family including human and rat 5-HT₁A receptors. Therefore, this residue is susceptible to account for the loss of a hydrogen bond in the complex of 5-HT with the mouse receptor. This loss would explain the low affinity of 5-HT for the mouse 5-HT₂B receptor. However, mouse wild-type and A79S 5HT₂B receptors displayed similar 5-HT bindings and transduction efficacies (Table II). Therefore, an involvement of Ala¹⁺⁵⁸(m⁷⁶) in 5-HT binding can be rejected. This conclusion is in agreement with the excessive distance between this TMD1 residue and the 5-HT molecule (8 Å be-
between the indolic nitrogen atom of 5-HT and the Cβ of Ala5.52(231) should not, therefore, participate to the mouse versus human 5-HT binding specificity.

T228A, P229A, A231T, and T228A/A231T Human 5-HT₂BRs—Significant differences in r.m.s.d. values between human and mouse 5-HT₂BR models, liganded or unliganded, could only be evidenced in the cases of the BR-based structures (Table I). Divergences in r.m.s.d. were observed mainly at the level of TMDs 5 and 6. We hypothesized that differences in the motions of these helices reflect their amino acid composition, in particular at the level of the amino acids in the vicinity of the Pro residues (29, 30). Bearing in mind that standard MD simulation algorithms do not allow simulations of the protein folding processes and that r.m.s.d. differences between human and mouse 5-HT₂BRs were not so large, we mutated Thr5.49(228) and Ala5.52(231) adjacent to Pro5.50(229) in TMD5 of the human 5-HT₂BR. The aim of this construction was to create a mouse-like sequence in this region (Fig. 1). Table II shows the resulting huge decrease (90–100-fold) of both the 5-HT binding affinity and the IP₃-coupling property of the obtained human double mutant receptor T228A/A231T. This mutant now exhibits characteristics similar to that of the mouse receptor with, however, a lower maximal 5-HT-induced IP₃ production (5-fold decrease). Similar effects on the receptor properties were observed with the single T228A (Kᵥ and EC₅₀ values are 90- and 20-fold decreased, respectively) and A231T (a 7-fold factor for both Kᵥ and EC₅₀) mutants. The P229A mutation was also performed. It only induced a 2-fold reduction in the expression level of the receptor. We therefore conclude that the specificities of 5-HT binding by the human or the mouse 5-HT₂BR are largely governed by different motions of TMDs 5 and 6.

**DISCUSSION**

In this report, three-dimensional computer modeling of the TMD bundle of human and mouse 5-HT₂BRs was undertaken on the basis of (i) sequence alignment (17), (ii) fitting to the BR and RH structures, and (iii) side-chain rotamer probability distributions (31). Based on the theoretical models obtained, mutated receptors were expressed and assessed for 5-HT binding affinity and 5-HT-induced IP₃ production. This functional approach allowed us to estimate the relevance of several predictions made on the basis of the sole computations of theoretical models and to propose structural models of 5-HT₂BRs (Figs. 3 and 4). Site-directed mutagenesis experiments also offered the possibility to go deeper into the mechanism of receptor activation (32) by giving particular attention to the conformational switch between the inactive and active states of the receptor. Finally, this work allowed us to define common fea-

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**Fig. 4.** Proposed transmembrane domain arrangements of BR-based models of human (a) and mouse (b) 5-HT₂BR receptors liganded with 5-HT.
properties between receptors of the 5-HT family and to consider the specificity of each 5-HTR in their capacity to accommodate the 5-HT ligand.

Modeled 5-HT2BR Structures Share Similarities with Other Members of the 5-HTR Family—As previously described in the 5-HT2AR (8, 33, 34) and 5-HT2CR (11) structures, the conserved Asp3.32 behaves as the primary counterion for the amino group of 5-HT in human and mouse 5-HT2Rs. The huge decrease observed upon mutation in both 5-HT binding and signaling efficacy parameters (Table II) confirms its interaction with the agonist in the activated state of the native forms of both human and mouse 5-HT2Rs.

Undoubtedly, Ser3.36 binds 5-HT in a way similar to that reported for the rat 5-HT2CR (11) and the human 5-HT2AR (9). Substitution of Ser by Ala did significantly decrease the affinity of human 5-HT2R for 5-HT (Table II). It is likely that Ser3.36 stabilizes 5-HT in the receptor binding site by H-bonding through the OH group of the neurotransmitter.

In the rat 5-HT2CR model (11), it was reported that Asn6.55 plays a role in the antagonist binding selectivity. In our proposed 5-HT2BR models, Asn6.55 is also likely to be involved in 5-HT binding. However, the nature of the interaction between this residue and the neurotransmitter depends on the template used for the modeling.

Despite controversial data in the case of the β2-adrenergic receptor (35), Asp2.50 and Asn7.49 were reported crucial for the interaction with most agonist molecules that bind to GPCRs and for the related couplings. This is especially clear with the human 5-HT2AR (28). In 5-HT2BRs, the 5-HT binding affinity and the signaling efficacy were similarly affected upon introduction of the D100N and N376D mutations. The double conservative mutation D100N/N376D restored the properties of the human 5-HT2BR to adopt an active conformation.

Consistent with this view, simultaneous substitution of either Pro5.50(h229) and both the 5-HT/2C receptors (21), this box maintains the 5-HT indole ring in a favorable orientation to interact with Asp3.32. The hydrophobic interaction between Phe6.52 and the indole ring of 5-HT is crucial for the geometry of the binding cavity. Upon 5-HT binding, a shift of Phe6.52(h341) induces a displacement of the Trp7.40(h337) side chain. Consequently, the other aromatic amino acids in the hydrophobic core of 5-HT2Rs are submitted to conformational changes.

In all 5-HT GPCRs, six conserved aromatic residues (Trp3.28, Phe3.35, Trp6.44, Trp7.40, and Trp7.49) were reported crucial for the binding of several neurotransmitters (5-HT, dopamine, and adrenaline) to their corresponding receptors. They also suggest the side chain conformations of these hydrophobic residues probably changed during the binding process. Such changes could directly affect the conformation of the adjacent helices, in particular in the vicinity of proline residues, and of other helices by propagation along the backbone of interacting conserved aromatic residues. Site-directed mutagenesis experiments also support the crucial role of the two above aromatic residues (36–38).

Human and Mouse 5-HT2BR Structures Display Distinct Aromatic Boxes—The BR- and RH-derived structural models of the human and mouse 5-HT2Rs cannot per se fully account for the 100-fold difference in 5-HT binding affinity observed between these two receptors. Upon 5-HT binding, as already suggested by Hibert et al. (7), the rearrangement of the hydrophobic aromatic core induces proline-mediated motions of TMDs 5 and 6. These motions are of smaller amplitude in the mouse 5-HT2R model than in the human receptor. Such differences may sustain the species specificity for 5-HT binding.
Thr5.49(m227) and the peptidic bond oxygen of Ala5.52(m230). This intra-helix interaction forms in the course of all MD simulations of the 5-HT/mouse 5-HT2B receptors. It may provide an explanation for the relatively lower r.m.s.d. values obtained for the mouse 5-HT2B three-dimensional models. This H bond may reduce the TMD5 motion amplitude and may constrain the aromatic network to accommodate 5-HT at its binding site. Finally, with the human double mutant, both the binding and the signaling parameters reach values similar to those measured with the wild-type mouse 5-HT2B.

In summary, the present study allowed us to predict that the 5-HT binding site of 5HT2B receptor primarily involves residues belonging to TMDs 3, 6, and 7. The BR-based structures appear more reliable than those RH-based for the modeling of 5-HT2B receptors. Indeed, the mutations of residues predicted to be in interaction with 5-HT according to the BR-based models systematically led to relevant results. With the help of site-directed mutagenesis, we show that 5HT2B receptors differ from 5HT2A receptors and are singular in the 5-HT2 receptor family. This work also provides a structural basis to explain the difference in 5-HT binding specificity between human and mouse 5-HT2B receptor. In conclusion, the enhanced structural knowledge on 5-HT2B may help in designing specific therapeutic drugs, which may reduce the TMD5 motion amplitude and may constrain the aromatic network to accommodate 5-HT at its binding site.


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The Serotonin Binding Site of Human and Murine 5-HT$_{2B}$ Receptors: MOLECULAR MODELING AND SITE-DIRECTED MUTAGENESIS
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