Molecular Dynamics Simulations of the Ligand-Induced Chemical Information Transfer in the 5-HT_{1A} Receptor

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Comparative molecular dynamics simulations of the 5-HT_{1A} receptor in its empty as well as agonist- (i.e. active) and antagonist-bound (i.e. nonactive) forms have been carried out. The agonists 5-HT and (R)-8-OH-DPAT as well as the antagonist WAY100635 have been employed. The results of this study strengthen the hypothesis that the receptor portions close to the E/DRY/W motif, with prominence to the cytosolic extensions of helices 3 and 6, are particularly susceptible to undergo structural modification in response to agonist binding. Despite the differences in the structural/dynamics behavior of the two agonists when docked into the 5-HT_{1A} receptor, they both exert a destabilization of the intrahelical and interhelical interactions found in the empty and antagonist-bound receptor forms between the arginine of the E/DRY sequence and both D133(3.49) and E340(6.30). For both agonists, the chemical information transfer from the extracellular to the cytosolic domains is mediated by a cluster of aromatic amino acids in helix 6, following the ligand interaction with selected amino acids in the extracellular half of the receptor, such as D116(3.32), S199(5.42), Y195(5.38), and F361(6.51). A significant reduction in the bend at P360(6.50), as compared to the empty and the antagonist-bound receptor forms, is one of the features of the agonist-bound forms that is related to the breakage of the interhelical salt bridge between the E/DRY arginine and E340(6.30). Another structural feature, shared by the agonist-bound receptor forms and not by the empty and antagonist-bound forms, is the detachment of helices 2 and 4, as marked by the movement of W161(4.50) away from helix 2, toward the membrane space.

INTRODUCTION

G protein-coupled receptors (GPCRs) constitute the largest superfamily of membrane proteins known to date.[1] They are also the primary sites of action of many of today life-saving drugs and the most promising targets for those to be developed in the future. Moreover, a growing number of spontaneous GPCR mutations that cause gain or loss of function have been found associated with serious inherited human disorders.[2]

Convergent and divergent mechanisms of evolution have built a blend of variance and conservation in this family of macromolecules. Conservation concerns their topology, i.e., seven α-helices that span the lipid bilayer and form a bundle when inserted in the membrane. This separates the N-terminus and three interconnecting loops exposed to the outside of the cell membrane from the C-terminus and an equal number of loops prospecting to the inside.[1] Also conserved is the basic strategy used to trigger a biological signal. Ligand binding promotes a conformational change that facilitates the interaction of the receptor with one or more members of the family of GTPases known as G proteins. In the inactive state, G proteins form membrane associated $\alpha\beta\gamma$-heterotrimers with GDP tightly bound to the α-subunit.[3] The activated receptor catalyzes the exchange of bound GDP for GTP, which dissociates the α-subunit from the $\beta\gamma$ dimer. The free subunits then interact with effector enzymes and ion channels, thus changing the levels of intracellular mediators.[3]

Despite the enormous biomedical importance of these receptors, the molecular mechanism, by which they are activated by ligand or mutation and recognize G proteins, still remains unknown. This frustrating situation is essentially due to the lack of high resolution structural information on these membrane proteins. So far, only the crystal structure of bovine rhodopsin has been resolved, and it has been made available at the end of year 2000.[4]

Over the last 10 years we have extensively used molecular modeling and computer simulations to infer the structural features characterizing the mutation- and ligand-induced inactive and active states of several GPCRs, including the α_{1b}-adrenergic, m3-muscarinic, oxytocin, and lutropin receptors.[5−15] Simulations on progressively refined receptor models all converged into the hypothesis that activating mutations and agonist binding exert a destabilizing effect on the interaction pattern of the arginine of the E/DRY sequence, thus increasing the solvent accessibility of selected amino acids at the cytosolic extensions of helices 3 and 6.[5−15]

In this study, we have performed molecular dynamics (MD) simulations on the 5-HT_{1A} receptor, another member of the rhodopsin family. This receptor has been the target of different molecular modeling studies by others, essentially aimed at mapping the ligand binding site often for drug

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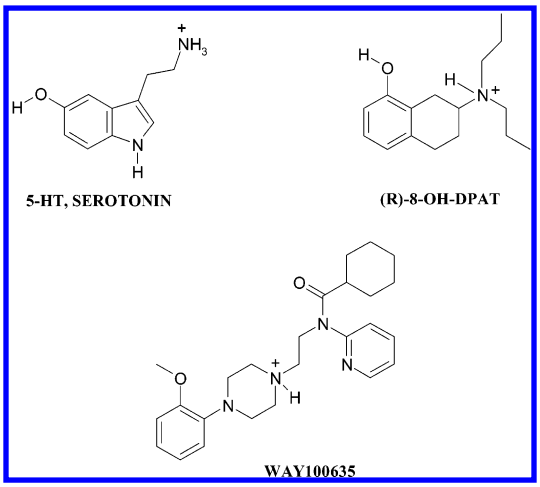
Chart 1



design purposes[16−21] and at comparing the structural changes induced by functionally different ligands.[22]

The three-dimensional (3D) model of the human 5-HT$_{1A}$ receptor herein presented has been achieved by comparative modeling, using the crystal structure of rhodopsin as a template.[4] Short (100 ps) and relatively long (2 ns) comparative MD simulations on the receptor in its empty as well as agonist- and antagonist-bound forms have been carried out, following the same computational approach as that previously employed for the ab initio models of the α_{1b}-adrenergic receptor (α_{1b}-AR) and m3-muscarinic receptor.[5] The agonists 5-hydroxy-tryptamine (5-HT or serotonin) and (R)-8-hydroxy-2-(di-*n*-propylamino)tetralin ((R)-8-OH-DPAT) as well as the antagonist N-{2-[4-(2-methoxyphenyl)-1-piperazinyl]-ethyl}-N-(2-pyridinyl) cyclohexanecarboxamide (WAY100635) have been considered (Chart 1).[23]

This study is essentially aimed at analyzing the structural effects exerted by functionally different ligands, docked into the extracellular half of the helix bundle, on the interaction pattern of the E/DRY arginine in the cytosolic domains.

METHODS

Comparative Modeling of the 5HT$_{1A}$ Receptor. The model of the human 5-HT$_{1A}$ receptor (SWISS-PROT Primary accession number P08908) was built by comparative modeling (by means of the MODELER program),[24] using the X-ray structure of rhodopsin as a template.[4] The receptor model holds the seven helices, the first, second, and third extracellular loops (e1, e2, and e3, respectively), the first and second intracellular loops (i1 and i2, respectively), and the short C-tail. Modeling of the N-terminus was skipped for the following reasons. In rhodopsin structure, a β-hairpin in the N-terminus interacts with a β-hairpin in e2, forming a β-sheet. Therefore, a strong structural relation between the N-terminus and e2 exists in the structure of the photoreceptor. In the 5-HT$_{1A}$ receptor, the N-terminal domain has the same length but no sequence similarity to the homologous domain of rhodopsin. Furthermore and most importantly, e2 significantly differs both in sequence and length from the corresponding domain of rhodopsin. Therefore, the same structural relation as that found in rhodopsin structure between the N-terminus and e2 is unlikely to occur in the 5-HT$_{1A}$ receptor. Thus, modeling the N-terminus of the 5-HT$_{1A}$ receptor by using, as a template, the homologous domain from rhodopsin structure might lead to unreliable models. On the same line, the huge i3 (i.e. 109 amino acids) was skipped because fold recognition methods did not suggest any reliable template for modeling this domain (results not shown).

A rhodopsin chimeric template was employed, in which e2 (i.e. sequence 177−199) was replaced by the homologous segment extracted from the ab initio model of the α_{1b}-AR in its input arrangement.[8,9] There are, indeed, similarities, concerning e2, between the 5-HT$_{1A}$ receptor and the α_{1b}-AR (Figure 1). The e2 of the α_{1b}-AR was built by subjecting to MD simulations the minimized coordinates of the isolated peptide in its extended conformation. Distance restraints were imposed between the backbone N-terminal nitrogen and C-terminal carbon atoms, probing different distance values, according to the distance between the C-terminal carbon atom of helix 4 and the N-terminal nitrogen atom of helix 5, in the input receptor model. Among the different average minimized structures obtained, the one was selected, which was able to properly bridge helices 4 and 5, allowing C195, in the N-terminal half of the loop, to form a disulfide bridge with C118(3.25) (for the amino acids in the helix bundle, the numbering from Ballesteros and Weinstein[25] is also reported in parentheses). This loop model is also able to properly bridge helices 4 and 5 in the rhodopsin template employed in this study.

Furthermore, the resolved portions of the rhodopsin's i3 (i.e. 226−235 and 240−248 segments) were deleted from the modified rhodopsin template.

The alignment reported in Figure 1 was employed for generating 25 different models of the 5-HT$_{1A}$ receptor, by randomizing the Cartesian coordinates of the model through a random number uniformly distributed in an interval from −4 Å to 4 Å. During the model building, α-helical restraints were imposed to the 218−227 and 336−343 amino acid stretches in the 5-HT$_{1A}$ receptor sequence in order to, respectively, prolong the C-terminal and N-terminal ends of helices 5 and 6 (Figure 1), similarly to the homology model of the α_{1b}-AR recently reported.[13] The refined receptor model was finally selected, which showed the lowest violation of spatial restraints (i.e. the lowest value of the Objective Function).[24] This model was also characterized by one of the highest 3D-Profile scores computed by means of the Protein Health module in the QUANTA 2000 package (www.accelrys.com). The selected model of the 5-HT$_{1A}$ receptor was completed by the addition of the polar hydrogens and was subjected to automatic and manual rotation of the side chain torsion angles, when in nonallowed conformation, leading to about 35 models.

Molecular Dynamics Simulations of the 5-HT$_{1A}$ Receptor in Its Empty and Ligand-Bound Forms. The models of the empty 5-HT$_{1A}$ receptor were subjected to in vacuo energy minimization and MD simulations by means of the CHARMM program.[26] Minimizations were carried out by using 1500 steps of steepest descent followed by a conjugate gradient minimization, until the root-mean-square gradient was less than 0.001 kcal/mol Å. A distance dependent dielectric term ($\epsilon = 4r$) was chosen. The "united atom approximation" was used.[26] MD simulations were carried out on the minimized coordinates of the 5-HT$_{1A}$ receptor in its free and ligand-bound forms. The lengths of the bonds involving the hydrogen atoms were restrained according to

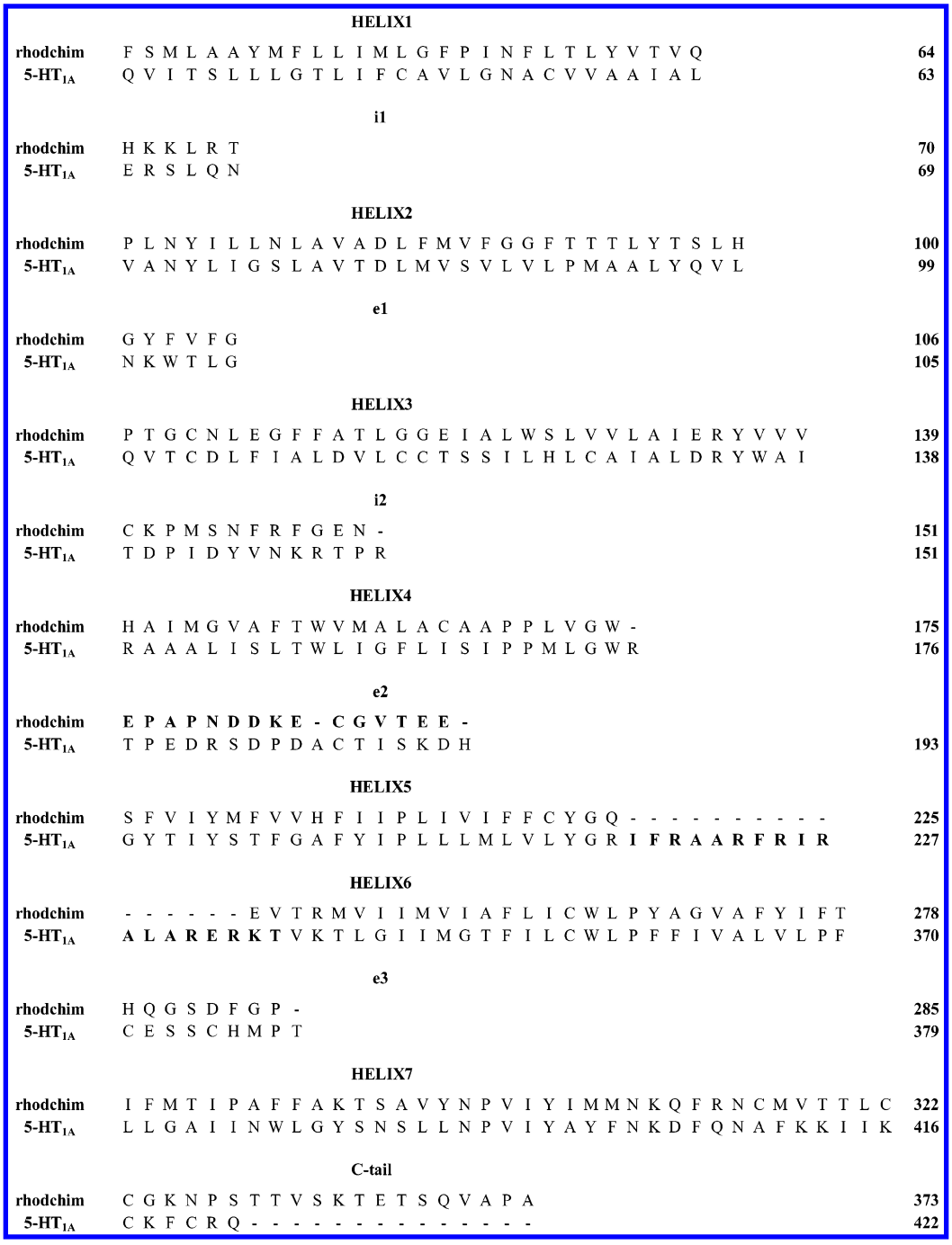


Figure 1. Sequence alignment between the rhodopsin chimeric template (rhodchim) and the human 5-HT$_{1A}$ receptor. The extracellular loops 1, 2, and 3 are, respectively, indicated as **e1**, **e2,** and **e3**, whereas the intracellular loops 1 and 2 are, respectively, indicated as **i1** and **i2**. In the modified template, e2 (i.e. the sequence 177−199) was substituted by the homologous segment extracted from the ab initio model of the α_{1b}-AR in its input arrangement[8] (bold characters on the rhodchim sequence). Moreover, the sequences 226−235 and 240−248 have been deleted. Bold characters on the 5-HT$_{1A}$ sequence concern the amino acids, which have been subjected to α-helical restraints by MODELER.

the SHAKE algorithm, allowing an integration time step of 0.001 ps. The systems were heated to 300 K with 5 K rise, every 100 steps per 6000 steps, by randomly assigning velocities from the Gaussian distribution. After heating, the system was allowed to equilibrate for 34 ps. A disulfide bridge patch was applied between C109(3.25) and C187 (in e2).

To account, at least in part, for the lack of the N-terminus and of i3, the backbone amino-functions of Q36(1.32) and A336(6.26) and the backbone carboxy-function of R227(5.80) were taken in their neutral state.

The secondary structure of the helix bundle was preserved, by assigning distance restraints (i.e. maximum and minimum allowed distances of 2.7 Å and 3.0 Å, respectively) between the backbone oxygen atom of residue i and the backbone nitrogen atom of residue i + 4. The application of these intrahelical distance restraints was instrumental in (a) reducing the system degrees of freedom, (b) inferring the structural/dynamics role of prolines, (c) compensating, at least in part, for the lack of the lipid environment, and (d) letting the helices move as rigid bodies, consistent with the experimental evidences on rhodopsin activation.[27]

For the empty 5-HT$_{1A}$ receptor form, short (100 ps) equilibrated MD runs were carried out, probing different input structures and different combinations of intrahelical distance restraints. The latter tests consisted in applying distance restraints to different amino acid stretches in each helix. Finally, the computation conditions and the input receptor structure were chosen, which, following MD simulation, produced an average arrangement characterized by a good quality (evaluated by means of the Protein Health module in the QUANTA 2000 package) together with structural similarity to rhodopsin. The similarity between the average minimized structures of the empty 5-HT$_{1A}$ receptor and rhodopsin structure was evaluated based upon the root-mean-square deviation (RMSD) of the main chain backbone atoms in the transmembrane domains as well as the degree of conservation of the interaction patterns involving the conserved amino acids. The selected average minimized structure of the 5-HT$_{1A}$ receptor showed a RMSD of 2.4 Å from rhodopsin structure. RMSD was computed by superimposing the main-chain atoms of segments 37−62, 74−100, 111−133, 152−171, 202−225, 252−276, and 286−306, representing the seven transmembrane domains of rhodopsin, with those in the homologous segments (i.e. 36−61, 74−99, 110−132, 152−171, 194−217, 344−368, and 380−400, respectively) of the 5-HT$_{1A}$ receptor model. These deviations are close to the expected value (i.e. between 1.5 Å and 2.2 Å), considering the sequence identity (24%) between the matched segments of the two receptors.[28] Moreover, the environment of highly conserved amino acids including N1.50, R3.50, F6.44, W6.48, and Y7.53 is rather conserved in the 5-HT$_{1A}$ receptor model and rhodopsin structure.

The intrahelical distance restraints, which led to the selected average arrangement of the empty 5-HT$_{1A}$ receptor, involve the following amino acid stretches: (a) 36−63 in helix 1, (b) 71−99 in helix 2, (c) 106−138 in helix 3, (d) 153−174 in helix 4, (e) 194−227 in helix 5, (f) 336−370 in helix 6, (g) 380−403 in helix 7, and (h) 406−416 in the receptor segment that corresponds to the rhodopsin's helix 8. The backbone oxygen atoms of the amino acids in position i-4 with respect to prolines as well as those of G202(5.45), A203(5.46), L356(6.46), W387(7.40), Y390(7.43), and N392(7.45) were not subjected to distance restraints. Indeed, in the input structure of the receptor, they do not accept H-bonds from the backbone NH-group in position i+4, similarly to rhodopsin structure. Furthermore, to preserve the noncanonical α-helical structure in the middle portion of helix 7, inherited from rhodopsin structure, distance restraints were imposed between the backbone oxygen atom of L388 and the amino acid in position i + 3.

The computation conditions and the input structure selected, based upon MD simulations of the empty 5-HT$_{1A}$ receptor, were used for simulating the ligand−receptor complexes, following manual docking of the agonists 5-HT and (R)-8-OH-DPAT as well as of the antagonist WAY100635. About 84 serotonin−receptor complexes were subjected to MD, which differed in the orientation of the ligand as well as in the conformations of the ligand and the receptor side chains involved in ligand interaction. Moreover, about 22 different values of distance restraints were probed between (a) the protonated nitrogen atom of the ligand and the carboxy-carbon atom of D116(3.32), and/or (b) either the hydroxy-hydrogen or oxygen atom of the ligand and, respectively, the hydroxy-oxygen or hydrogen atom of S199(5.42), or (c) the hydroxy-oxygen atom of the ligand and the hydroxy-oxygen atom of S199(5.42). About 8 different (R)-8-OH-DPAT−receptor complexes were simulated, testing 28 different values of distance restraints, involving the same atom pairs as those that were considered for the simulations of the serotonin−receptor complexes. Finally, 5 different WAY100635−receptor complexes were simulated, testing 15 different values of distance restraints applied between the protonated nitrogen atom of the ligand and the carboxy-carbon atom of D116(3.32). The amino acids D116(3.32) and S199(5.42) and their homologues have been, indeed, demonstrated to play an important role in ligand binding and in function of the 5-HT$_{1A}$ and other serotonin−receptor subtypes.[29,30] For some selected agonist- and antagonist−receptor complexes, MD runs without using intermolecular distance restraints were also carried out.

Tests were done by using equilibrated MD runs of 100 ps. The computation conditions (i.e. input structures and intermolecular distance restraints), which led to average arrangements of the ligand−receptor complexes characterized by good quality (checked by means of the Protein Health module in the QUANTA 2000 package) and consistency with the available experimental data (see Results), were used for generating 2 ns trajectories.

The structures of serotonin and WAY100635 were built by means of the QUANTA 2000 package (http://www.accelrys.com), whereas the initial structure of the (R)-8-OH-DPAT was downloaded from the Cambridge Structure Databank (http://www.ccdc.cam.ac.uk). The three ligand structures, in their protonated forms, were optimized by using the semiempirical MO AM1 calculation within the MOPAC 6.0 (QCPE 455) suite of programs.[31] The phenyl-ring of WAY100635 was oriented according to the geometries of the energy minima as previously inferred from a conformational analysis on substituted phenylpiperazines.[32]

RESULTS

This study is aimed at finding structural relations between intermolecular interactions in the extracellular half of the 5-HT$_{1A}$ receptor, as made by activating (i.e. 5-HT and (R)-8-OH-DPAT) and nonactivating (i.e. WAY100635) ligands, and intramolecular interactions in the cytosolic domains, focusing on the interaction pattern of the functionally important arginine of the E/DRY sequence.[1] The approach consists of comparative analyses of a large number of short (100 ps) and relatively long (2 ns) MD trajectories, probing different starting structures as well as different intrahelical (for the empty receptor form) and intermolecular (for the ligand-bound forms) distance restraints. In particular, the following numbers of equilibrated MD runs have been carried out: (a) about 40 short and 1 long for the empty 5-HT$_{1A}$ receptor, (b) 250 short and 5 long for the serotonin−receptor complexes, (c) about 71 short and one long for the (R)-8-OH-DPAT−receptor complex, and (d) about 60 short and 3 long for the WAY100635−receptor complexes.

For the empty 5-HT$_{1A}$ receptor, the criteria for selecting the short trajectory that could be worth prolonging included the quality of the average minimized structures and their degree of similarity to rhodopsin structure (see Methods).

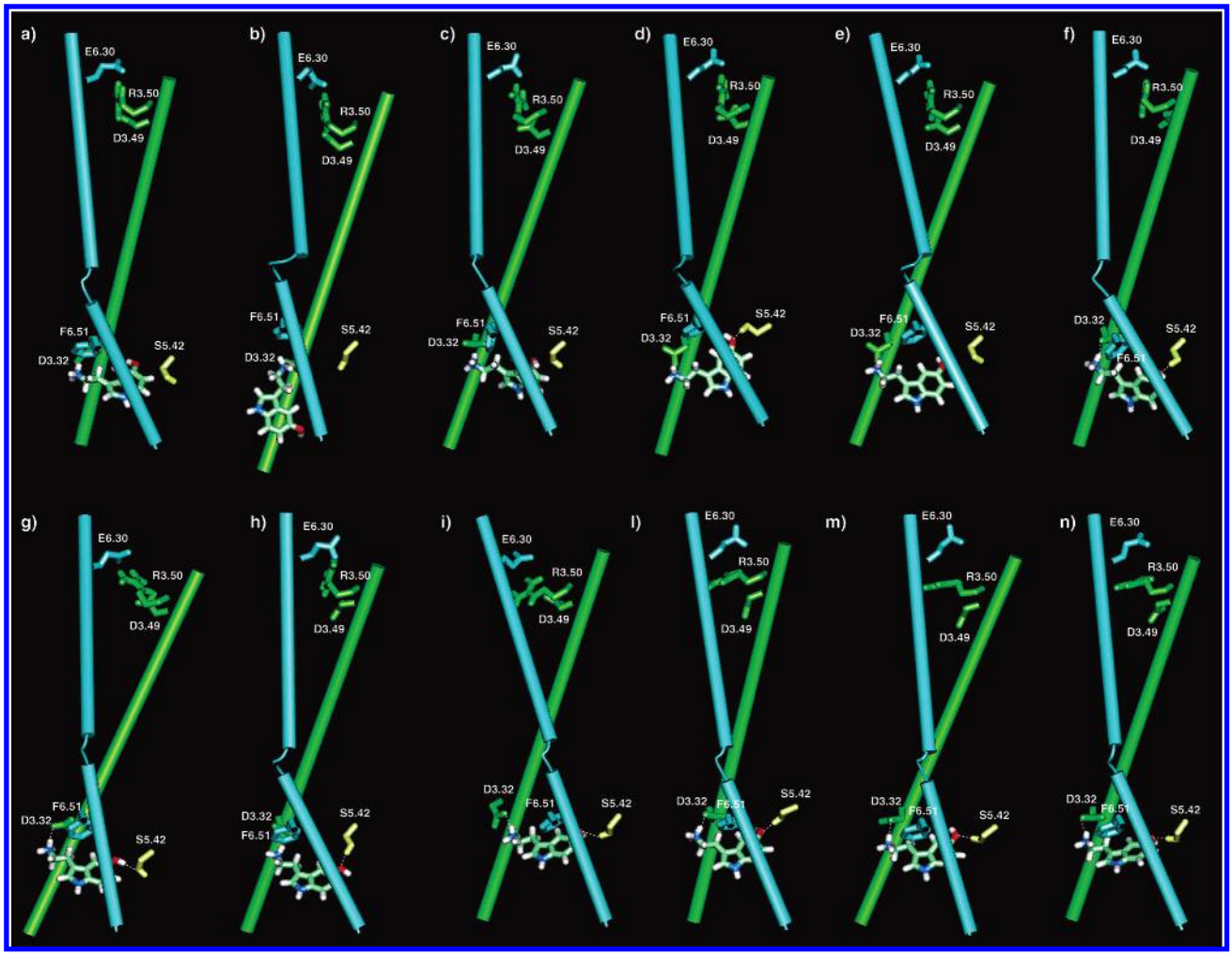


Figure 2. Average minimized structures of the serotonin−receptor complexes averaged over the 200 structures collected during the equilibrated 100 ps time of different MD simulations, which differed only for the conditions of intermolecular distance restraints. In detail, the structure represented in panel **a** is the outcome of MD simulations without using intermolecular distance restraints. For the structures shown in panels **b** and **c**, distance restraints have been applied between the protonated nitrogen atom of the ligand and the carboxy-carbon atom of D116(3.32), by, respectively, using minimum and maximum allowed distances of 3.0 Å and 3.5 Å (**b**); and of 3.0 Å and 3.2 Å (**c**). For the structures shown in panels **d**, **f**, and **m**, distance restraints have been applied between the hydroxy-oxygen atom of the ligand and the hydroxy-hydrogen atom of S199(5.42), by, respectively, using minimum and maximum allowed distances of 1.8 Å and 2.0 Å (**d**); of 1.9 Å and 2.0 Å (**f**); and of 1.8 Å and 2.0 Å (**m**). For the structure shown in panel **e**, distance restraints have been applied between the hydroxy-oxygen atom of the ligand and the hydroxy-oxygen atom of S199(5.42), by using minimum, respectively, and maximum allowed distances of 2.9 Å and 3.0 Å. For the structure shown in panel **g**, distance restraints have been applied between the protonated nitrogen atom of the ligand and the carboxy-carbon atom of D166(3.32) (i.e. minimum and maximum allowed distances of, respectively, 2.5 Å and 3.0 Å) and between the hydroxy-oxygen atom of the ligand and the hydroxy-oxygen atom of S199(5.42) (i.e. minimum and maximum allowed distances of, respectively, 2.9 Å and 3.0 Å). For the structures shown in panels **h**, **i**, **l**, and **m**, distance restraints have been applied between the protonated nitrogen atom of the ligand and the carboxy-carbon atom of D166(3.32) (i.e. minimum and maximum allowed distances of, respectively, 3.0 Å and 3.5 Å (**h**); of 2.7 Å and 3.0 Å (**i**); of 2.9 Å and 3.0 Å (**l**); and of 2.5 Å and 3.0 Å (**m**)) and between the hydroxy-oxygen atom of the ligand and the hydroxy-hydrogen atom of S199(5.42) (i.e. minimum and maximum allowed distances of 1.9 Å and 2.1 Å, respectively, for the structure in panel **h** and of 1.8 Å and 2.0 Å, respectively, for the structures panels in **i**, **l**, and **m**. The models are viewed in a direction parallel to the membrane surface, the cytosolic side being at the top. Only helices 3 and 6 have been shown, represented by cylinders. Details of the interactions made by R134(3.50) and by serotonin are also shown. The amino acid side chains are colored according to their locations: helices 1, 2, 3, 4, 5, 6, and 7 are, respectively, colored in blue, orange, green, pink, yellow, cyan, and violet. The intermolecular H-bonds are indicated by manually drawn dashed lines. The amino acids are labeled according to the numbering scheme proposed by Ballesteros and Weinstein.[25]

For the ligand−receptor complexes, the criteria for selecting the trajectories that could be worth prolonging included the quality of the average minimized structures as well as their consistency with the results of site-directed mutagenesis experiments, targeting the putative ligand binding site of the 5-HT receptors[29,30,33] and of a close homologous GPCR, such as the α_{1b}-AR.[34,35] As an example, Figure 2 shows details of 12 average minimized complexes between serotonin and the 5-HT_{1A} receptor. These complexes are the outcome of 100 ps MD runs, which differed only in the intermolecular distance restraints that have been applied (for details see the legend to Figure 2). The computation conditions, which led to the serotonin−receptor complexes shown in panels **i**, **l**, **m**, and **n** (Figure 2), have been used for producing 2 ns equilibrated trajectories. The choice of these complexes has been dictated, at least in part, by the singular occurrence of all-together the intermolecular interactions that are thought to be crucial for agonist function: (a) the charge-reinforced H-bond between the protonated nitrogen atom of the ligand and D116(3.32); (b) the H-bond between the hydroxy-group

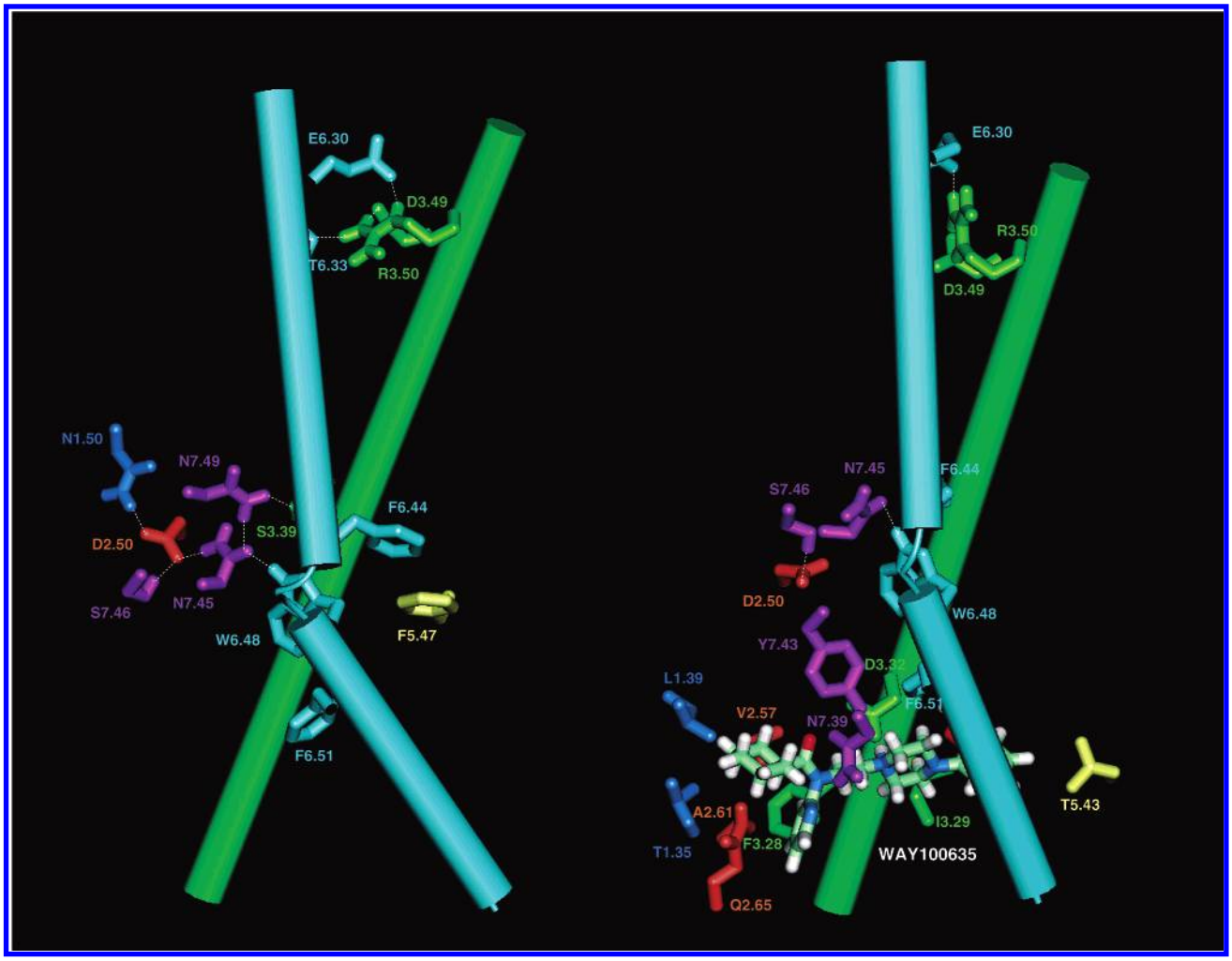


Figure 3. Structures of the empty (left) and the WAY100635-bound (right) forms of the 5-HT$_{1A}$ receptor averaged over the 1000 structures collected during the last 500 ps of a 2 ns trajectory and minimized. The models are viewed in a direction parallel to the membrane surface, the cytosolic side being at the top. Only helices 3 and 6 have been shown, represented by cylinders. Details of the interactions made by R134(3.50) and by the antagonist are also shown. Moreover, only the polar conserved amino acids that are involved in H-bonds (indicated by manually drawn dashed lines) are shown. Color coding and amino acid numbering follow the same criteria as those that have been described in the legend to Figure 2.

of the ligand and S199(5.42), and (c) van der Waals interactions between the indole ring of the ligand and F361-(6.51) (Figure 2). The first two interactions are consistent with the results of mutagenesis experiments on the 5-HT$_{1A}$ receptor,[29] whereas the latter is consistent with the suggestions from biochemical experiments on the α_{1b}-AR.[34]

The mechanistic hypothesis inferred from this study is based upon the outcome of all the short and long MD runs that have been carried out. However, the presentation of the results will be focused on one rather representative long trajectory for each of the four receptor forms considered in this study (i.e. free as well as serotonin- (R)-8-OH-DPAT- and WAY100635-bound forms).

Structural Features of the Empty 5-HT$_{1A}$ Receptor and Its Antagonist-Bound Form. A characteristic feature of the empty 5-HT$_{1A}$ receptor structure averaged over the 1000 structures collected during the last 500 ps of the selected MD trajectory is the salt bridge interaction between R134-(3.50) of the E/DRY sequence, at the cytosolic extension of helix 3, and both the adjacent aspartate (i.e. D133(3.49)) and E340(6.30) (Figure 3). The two salt bridge interactions, which were already present, thought weaker, in the input structure, are persistent during the 2 ns time of MD simulation (Figure 4). However, the intrahelical salt bridge is more stable than the interhelical one, because the charge-reinforced H-bond between R134(3.50) and the adjacent aspartate is conserved during the whole 2 ns simulation, whereas that between R134(3.50) and E340(6.30) is alternately lost and gained. The loss of the H-bonding contribution to this interhelical interaction is always concurrent with the establishment of another H-bonding interaction between the E/DRY arginine and T343(6.33).

Furthermore, differently from the input structure, in the average minimized structure of the empty 5-HT$_{1A}$ receptor, the polar conserved amino acids in the transmembrane (TM) portions of helices 1, 2, 3, and 7 form a network of H-bonding interactions, interconnecting the middle portions of helices 1, 2, 3, 6, and 7 (Figure 3). In particular, (a) N54(1.50) interacts with D82(2.50), (b) D82(2.50) interacts also with N392(7.45) and S393(7.46), (c) N392(7.45) interacts also with both W358(6.48) and N396(7.49), and (d) N396(7.49) interacts also with S123(3.39) (Figure 3). These interactions, with the exception of that between N54(1.50) and D82(2.50) and that between N396(7.49) and S123(3.39), are rather persistent during the 2 ns simulation. However, we do not exclude the possibility that one or more water

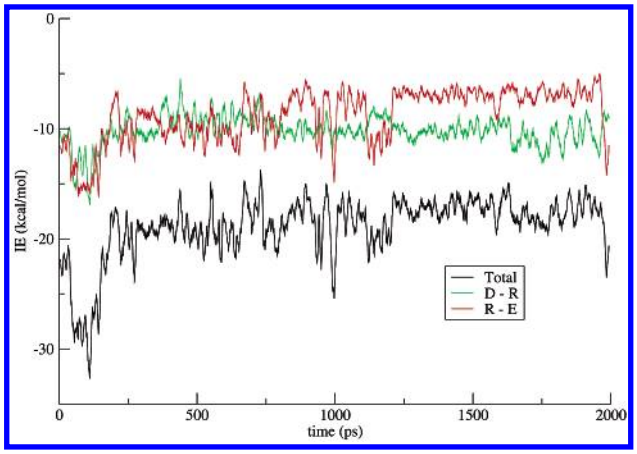


Figure 4. Plot of the interaction energies (kcal/mol) versus time concerning the interacting pairs R134(3.50)-D133(3.49) (green line, D−R) and R134(3.50)-E340(6.30) (red line, R−E) in the empty 5-HT_{1A} receptor. The summation over the two interaction energies (black line, total) has also been plotted. Lines have been smoothed by making running averages on 20 points, through the program Xmgrace.

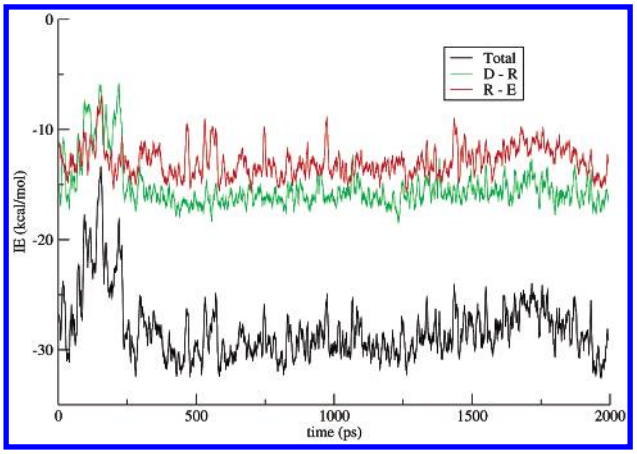


Figure 5. Plot of the interaction energies (kcal/mol) versus time concerning the interacting pairs R134(3.50)-D133(3.49) (green line, D−R) and R134(3.50)-E340(6.30) (red line, R−E) in the WAY 100635−receptor complex. The summation over the two interaction energies (black line, total) has been also plotted. Lines have been smoothed by making running averages on 20 points, through the program Xmgrace.

molecules might mediate the H-bonding network involving these polar conserved amino acids. This is also suggested by the advances in determination of rhodopsin structure.[36]

Another feature that characterizes the empty receptor form is the interaction between W161(4.50) and S77(2.45). The latter amino acid is homologous to N2.45, that in rhodopsin structure is, indeed, the interaction partner of the highly conserved tryptophan in helix 4.[4,36] The nature of the S77-(2.45)−W161(4.50) interaction is van der Waals attractive for great part of the simulation, an H-bond occurring only in the early steps.

In the WAY100635−receptor complex averaged over the 1000 structures collected during the last 500 ps of the selected MD trajectory, the protonated nitrogen atom of the ligand performs a charge-reinforced H-bond with D116(3.32) (Figure 3). This interaction persists during the entire MD simulation, due also to the distance restraints (i.e. minimum and maximum allowed distances of 3.4 Å and 3.5 Å, respectively) that have been imposed between the protonated nitrogen atom of the ligand and the carboxy-carbon atom of D116(3.32). The importance of this interaction for the binding of WAY100635 at the 5-HT_{1A} receptor has been inferred from indirect experimental evidences.[30]

In agreement with the results of automatic docking of the 2-methoxyphenylpiperazine fragment into the 5-HT_{1A} receptor binding site (results not shown), the 2-methoxyphenylpiperazine of the antagonist docks into a site formed by amino acids of helices 3, 4, 5, and 6 as well as of e2. Due to the geometrical constraints imposed by the intermolecular charge-reinforced H-bonding interaction, the remaining part of the ligand mainly docks into a site formed by helices 1, 2, 3, and 7 (Figure 3). In particular, a very weak H-bond occurs between the pyridine nitrogen atom of the ligand and the side chain nitrogen atom of N389(7.39), the distance between these two heteroatoms being 4 Å, on average. Moreover, N389(7.39) is involved in weak van der Waals interactions with the cyclohexyl-ring of the ligand. The intermolecular interaction mode of N389(7.39) is consistent with site directed mutagenesis data showing that the valine substitution for N389(7.39) induces only a 2.3-fold reduction in the 5-HT_{1A} binding affinity of WAY100635.[33] The other interactions, which frequently occur during the 2 ns time of simulation, include the following: (a) van der Waals interactions between the phenyl-ring of the ligand and both T200(5.43) and A365(6.55) of the receptor, (b) van der Waals interactions between the piperazine ring of the ligand and I113(3.29) of the receptor, (c) van der Waals interactions between the pyridine ring of the ligand and both Q97(2.65) and F112(3.28) of the receptor, and (d) van der Waals interactions between the cyclohexyl-ring of the ligand and T39(1.35), L43(1.39), V89(2.57), and A93(2.61) of the receptor (Figure 3).

Due to the geometry of the intermolecular charge-reinforced H-bond, the rather rigid antagonist maintains almost the same orientation, conformation, and interaction pattern over whole 2 ns simulation. This intermolecular interaction mode of WAY100635 is associated with persistent intramolecular charge-reinforced H-bonds between the E/DRY arginine and both D133(3.49) and E340(6.30) (Figures 3 and 5). Due to the persistency of the H-bonding contribution to these intrahelical and interhelical salt bridges, the mobility of the E/DRY arginine side chain is significantly reduced in the antagonist-bound form, as compared to the empty 5-HT_{1A} receptor (Figures 3−5).

The antagonist binding, on the other hand, destabilizes the majority of the H-bonding interactions that, in the empty receptor, interconnect the polar conserved amino acids in helices 1, 2, 3, and 7 (Figure 3).

Structural Features of the Agonist−Receptor Complexes. In the selected MD trajectory of the serotonin−receptor complex, the ligand is constrained to interact with both D116(3.32) and S199(5.42). Indeed, distance restraints have been applied between (a) the protonated nitrogen atom of the ligand and the carboxy-carbon atom of D116(3.32) (i.e. minimum and maximum allowed distances of 2.5 Å and 3.0 Å, respectively) and (b) the hydroxy-oxygen atom of the ligand and the hydroxy-hydrogen atom of S199(5.42) (i.e. minimum and maximum allowed distances of 1.8 Å and 2.0 Å, respectively). These amino acids have been shown to be important for serotonin binding by site directed mutagenesis

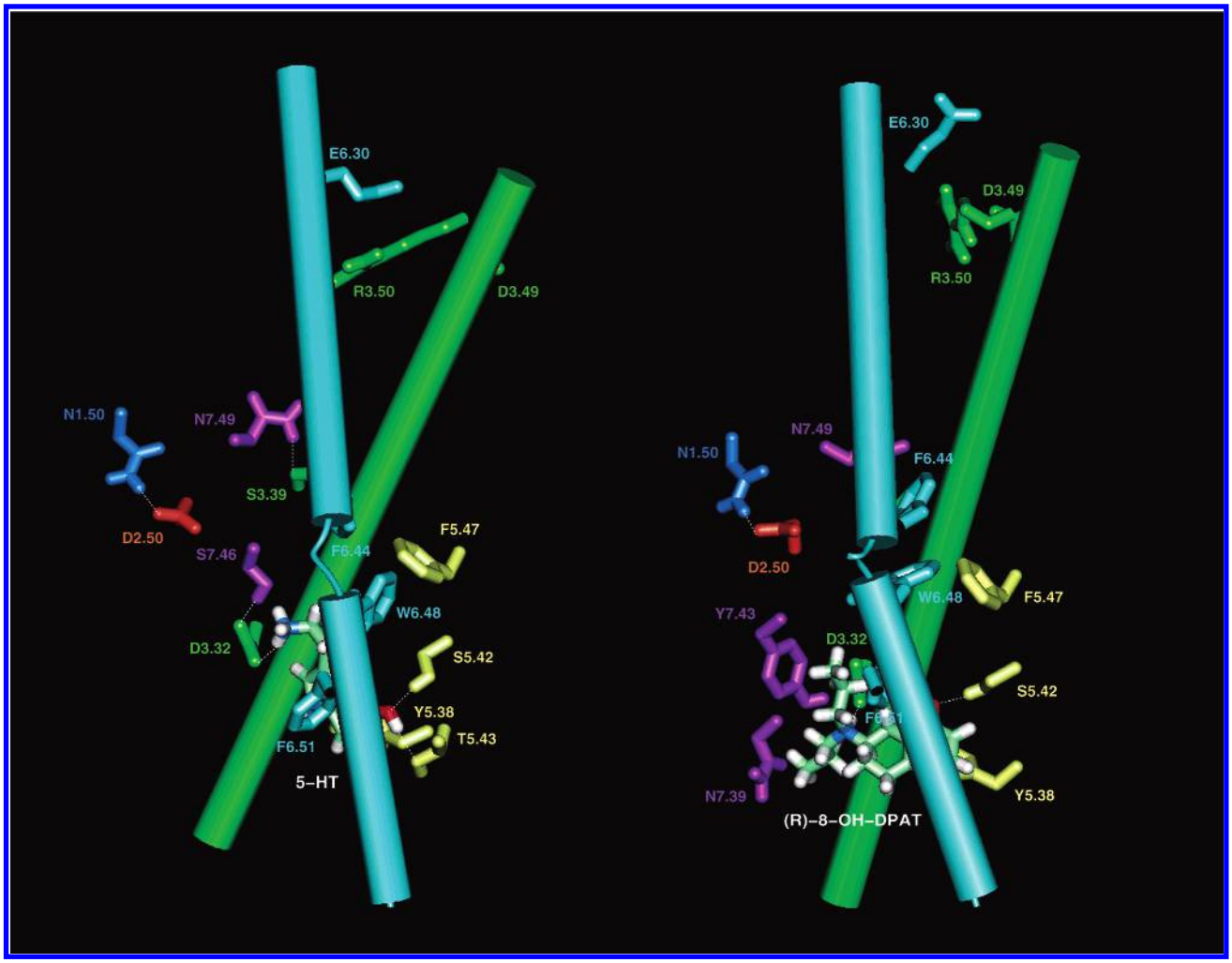


Figure 6. Structures of the 5-HT-bound (left) and (R)-8-OH-DPAT-bound (right) forms of the 5-HT$_{1A}$ receptor averaged over the 1000 structures collected during the last 500 ps of a 2 ns trajectory and minimized. Graphic representations, color coding, and amino acid numbering follows the same criteria as dose described in the legends to Figures 2 and 3.

experiments.[29] For a great part of the simulation time, the indole ring of the ligand stays almost perpendicular with respect to the putative membrane surface, being involved in interaction with the aromatic rings of both Y195(5.38) and F361(6.51). Moreover, during the last 500 ps of MD simulation, the hydroxy-group of the agonist is found involved in an additional H-bond with T200(5.43) (Figure 6). This interaction mode of the agonist into the extracellular half of the helix bundle is concurrent with the breakage of the intrahelical salt bridge found, in the empty and the antagonist-bound receptor forms, between the arginine of the E/DRY sequence and the adjacent aspartate (Figures 3, 6, and 7). The latter performs a new salt bridge with K147 in i2. As for the interhelical interaction between R134(3.50) and E340(6.30), the H-bonding contribution is permanently lost during the entire simulation, whereas the electrostatic component is significantly dropped in about the first 400 ps and the last 700 ps (Figure 7). Over the whole MD simulation of the serotonin−receptor complex, E340(6.30) is involved in an intrahelical salt bridge with the adjacent R341(6.31) or, less frequently, in H-bonding interactions with Q68 and N69 in i1.

The breakage of the R134(3.50)−E340(6.30) salt bridge in the cytosolic extensions of helices 3 and 6 is concurrent with the strengthening of the interaction between the indole ring of the ligand and F361(6.51), in the extracellular half of the helix bundle (Figures 2 and 6).

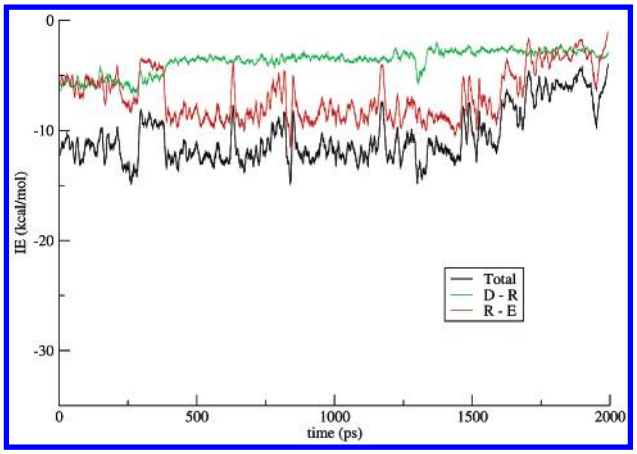


Figure 7. Plot of the interaction energies (kcal/mol) versus time concerning the interacting pairs R134(3.50)-D133(3.49) (green line, D−R) and R134(3.50)-E340(6.30) (red line, R−E) in the serotonin−receptor complex. The summation over the two interaction energies (black line, total) has been also plotted. Lines have been smoothed by making running averages on 20 points, through the program Xmgrace.

Furthermore, the serotonin-bound receptor form is characterized by the breakage of the H-bonding network made,

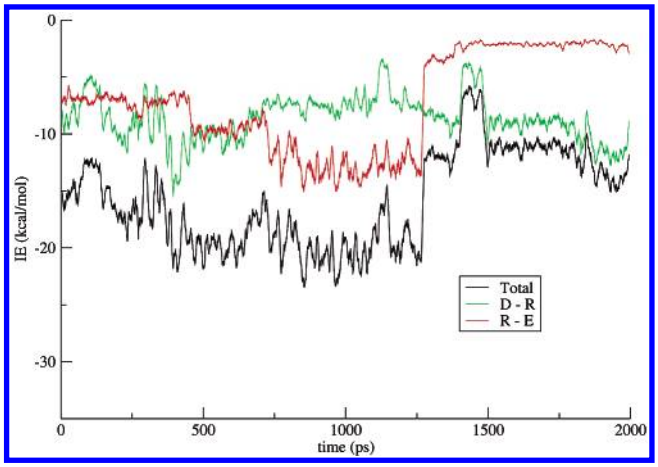


Figure 8. Plot of the interaction energies (kcal/mol) versus time concerning the interacting pairs R134(3.50)-D133(3.49) (green line, D−R) and R134(3.50)-E340(6.30) (red line, R−E) in the (R)-8-OH-DPAT−receptor complex. The summation over the two interaction energies (black line, total) has been also plotted. Lines have been smoothed by making running averages on 20 points, through the program Xmgrace.

in the empty receptor, by the side chains of the polar conserved amino acids in helices 1, 2, 3, and 7 (Figures 3 and 6). Moreover, a detachment between helices 2 and 4 occurs in the serotonin-bound form, as compared to the empty receptor, concurrent with an outward movement of helix 3. This structural effect is marked by the highly conserved W161(4.50) that moves away from helix 2 toward the membrane space, loosing the interaction with S77(2.45) that characterizes the empty receptor structure. Indeed, in the empty 5-HT_{1A} receptor structure averaged over the 1000 structures collected during the last 500 ps of the selected MD trajectory, the distance between the α-carbon atoms of S77(2.45) and W161(4.50) is 7.5 Å, whereas in the corresponding average minimized structure of the serotonin−receptor complex this distance is 10.5 Å.

In the 2 ns MD trajectory of the (R)-8-OH-DPAT−receptor complex, the agonist is constrained to interact with both D116(3.32) and S199(5.42), assuming that these two amino acids, which are important for serotonin function,[29,30] are relevant for (R)-8-OH-DPAT as well. Indeed, distance restraints have been applied between (a) the protonated nitrogen atom of the ligand and the carboxy-carbon atom of D166(3.32) (i.e. minimum and maximum allowed distances of 3.4 Å and 3.5 Å, respectively) and (b) the hydroxy-oxygen atom of the ligand and the hydroxy-hydrogen atom of S199-(5.42) (i.e. minimum and maximum allowed distances of 1.8 Å and 2.0 Å, respectively). Under these conditions, the ligand induces fluctuations in both the salt bridge interactions that involve R134(3.50) in the empty receptor (Figure 8). In particular, the charge-reinforced H-bond between the E/DRY arginine and the adjacent aspartate is alternately gained and lost, respectively, strengthening and weakening the salt bridge. Similar fluctuations are also observed for the R134-(3.50)−E340(6.30) interaction. The strengthening of the D133(3.49)−R134(3.50) interaction is concurrent with the weakening of the R134(3.50)−E340(6.30) interaction and vice versa (Figure 8). Interestingly, the interhelical salt bridge is definitely lost in the last 500 ps of simulation (Figures 6 and 8), E340(6.30) being involved in an intrahelical salt bridge with R341(6.31). Similarly to the serotonin−receptor complex, the breakage of the R134(3.50)−E340(6.30) interaction in the cytosolic side is concurrent with the strengthening of the interaction between the tetralin ring of the ligand and F361(6.51) of the receptor, in the extracellular side (Figure 6).

The tetralin ring of the ligand stays almost perpendicular with respect to the putative membrane surface during the whole 2 ns simulation, performing van der Waals interactions with both Y195(5.38) and A365(6.55) (Figure 6). Furthermore, one of the two propyl-chains of the ligand performs van der Waals interactions with both I113(3.29) and W67 (in e1), whereas the other makes van der Waals interactions with both F361(6.51) and Y390(7.43) (Figure 6). The orientation of the tetralin ring of the ligand in the (R)-8-OH-DPAT−receptor complexes inferred from this study appears to be similar to that recently proposed by Sylte et al.,[22] the main differences residing in the orientation of the two propyl-chains.

Similarly to the serotonin−receptor complex, the (R)-8-OH-DPAT−receptor complexes are characterized by the breakage of the H-bonding network that, in the empty receptor, involves the polar conserved amino acids (Figures 3 and 6). Moreover, W161(4.50) moves away from helix 2 toward the putative membrane space, similarly to the serotonin-bound receptor form.

Structural/Dynamics Differences between 5-HT- and (R)-8-OH-DPAT−Receptor Complexes. The dynamics behavior of serotonin and (R)-8-OH-DPAT is different and correlates, at least in part, with the observation that, differently from the natural agonist, (R)-8-OH-DPAT is selective for the 5-HT_{1A} receptor, as compared to the other 5-HT receptor subtypes.[23] Indeed, serotonin fluctuates in orientation and conformation much more than (R)-8-OH-DPAT during MD simulation. As a consequence, the interaction pattern of serotonin changes during simulations, i.e., H-bonding interactions can also occur, even if less frequently than the interactions mentioned above, between (a) the indole NH-group of the ligand and T188 (in e2) and (b) the protonated nitrogen atom of the ligand and Y390-(7.43) or S393(7.46). In contrast, the orientation and interaction pattern of (R)-8-OH-DPAT are rather constant over the 2 ns simulation. The higher mobility of serotonin, as compared to (R)-8-OH-DPAT, might be suggestive of a higher ability of the natural agonist to fit into the binding sites of the different 5-HT receptor subtypes.

Differences between serotonin and (R)-8-OH-DPAT also concern the interaction pattern of the E/DRY arginine (Figures 7 and 8), in their complexes with the 5-HT_{1A} receptor. In particular, the last 500 ps of the selected MD trajectory of the serotonin−receptor complex are characterized by the breakage of both the intrahelical and interhelical salt bridges made by the E/DRY arginine, whereas only the interhelical salt bridge is broken in the corresponding simulation period of the (R)-8-OH-DPAT−receptor complex (Figures 7 and 8). The different dynamics behavior of serotonin and (R)-8-OH-DPAT, as shown by our computations, is in line with the experimental observations that the former is indeed a full agonist, whereas the latter is a full agonist in some biochemical assays and a partial agonist in others.[37]

DISCUSSION

In this study, we have essentially employed the same strategy that we used 10 years ago to infer the structural modifications induced by functionally different ligands (agonists and antagonist) upon docking to the α_{1b}-adrenergic and m3-muscarinic receptors.[5] The approach consisted into manual docking of selected agonists and antagonists into the putative binding sites of the input receptor structures, followed by comparative MD simulations. The main differences between the previous and the present studies concern (a) the receptor systems, (b) the way in which the starting receptor models have been achieved, (c) the receptor domains included in the 3D models, and (d) the length of MD simulations. Indeed, in the previous study, the receptor models were built by ab initio modeling,[5] whereas, in the present work, comparative modeling has been carried out taking advantage of the availability of rhodopsin structure.[4] Furthermore, the previous models included only the transmembrane helices, whereas the 5-HT$_{1A}$ receptor model employed in this study includes also the three extracellular loops, the first and second intracellular loops, and the C-tail. Finally, a significantly higher number of MD simulations, including also 2 ns runs, have been carried out in the present study as compared to the previous ones.[5] Despite the differences between previous and present approaches, hallmarks of the ligand-induced active and nonactive forms of the α_{1b}-adrenergic, m3-muscarinic, and 5-HT$_{1A}$-serotoninergic receptors have been found to involve R3.50, the arginine of the E/DRY motif and the cytosolic extensions of helices 3 and 6. In fact, in this study, we have found that, in the empty 5-HT$_{1A}$ receptor, R3.50 is involved in salt bridge interactions with both D133(3.49) and E340(6.30). Equivalent interactions are found between R135(3.50) and both E134(3.49) and E247(6.30) in the structure of the inactive state of rhodopsin.[4] The analysis of the selected MD trajectory suggests that the strength of the interhelical interaction is lower and fluctuates more than that of the intrahelical one. This is due to the loss/gain of the H-bonding contribution to the interhelical salt bridge during the 2 ns simulation time. These results are consistent with the advances in determination of rhodopsin structure that show the lack of the charge-reinforced H-bond between R135(3.50) and E247(6.30), whereas the H-bond between the conserved arginine and the adjacent glutamate is retained, as compared to earlier structural determinations.[4,36] Our results also agree with the high B-factor of E247(6.30) found in the structure determinations of rhodopsin and suggestive of high mobility of its side chain.[4,36] Molecular simulations suggest that the loss of the H-bonding contribution to the R134(3.50)−E340(6.30) interhelical interaction is concurrent with the establishment of an H-bonding interaction between the same conserved arginine in helix 3 and T343(6.33). In summary, in the empty 5-HT$_{1A}$ receptor, the cytosolic extensions of helices 3 and 6 are permanently linked by H-bonding interactions between R134(3.50) and either E340(6.30) and/or T343(6.33). Thus, D133(3.49), E340(6.30), and T343(6.33) might contribute to keeping the 5-HT$_{1A}$ receptor in its inactive state, by constraining the motion of the conserved E/DRY arginine. The role of D133(3.49) in stabilizing the inactive state of the 5-HT$_{1A}$ receptor is supported by the findings that the irreversible charge neutralization (following mutation) of the glutamate/aspartate in the homologous position induces the constitutive activation of several GPCRs of the rhodopsin family, including rhodopsin.[6,7,38−40] Furthermore, computations and experiments have suggested that, similarly to rhodopsin, the salt bridge between the amino acids homologous to R134(3.50) and E340(6.30) of the 5-HT$_{1A}$ is one of the intramolecular interactions characterizing the inactive states of the β_2- and α_{1b}-adrenergic, the 5-HT$_{2A}$-serotoninergic, and the lutropin receptors.[13−15,41−43] The additional involvement of T343(6.33) in stabilizing the inactive state of the 5-HT$_{1A}$ receptor agrees with the hypothesis that T279(6.34) would substitute E6.30 as interacting partner of R3.50 in the μ opioid receptor.[44] Indeed, the positions of these threonines in the 5-HT$_{1A}$ and the μ opioid receptors are adjacent, both facing the arginine of the E/DRY motif.

Molecular simulations suggest that the establishment of strong and persistent charge-reinforced interactions between the protonated nitrogen atom of the antagonist WAY100536 and D116(3.32) of the receptor is concurrent with the stabilization of both the intrahelical and interhelical salt bridges that, in the empty receptor form, involve the E/DRY arginine, thereby strengthening the computation- and experiment-based hypothesis that these interactions are features of the inactive receptor states.[4,6,7,36,38−43]

The analysis of an extensive number of different MD trajectories suggests that the interactions with three relevant amino acids of the putative ligand binding site, in the extracellular half of the helix bundle, are essential for both agonists to destabilize the salt bridge interactions that, in the empty and antagonist-bound receptor forms, involve the E/DRY arginine (Figure 2). These amino acids are (a) D116(3.32) that recognizes the protonated nitrogen atom of the ligand, (b) S199(5.42) that interacts with the indole or tetralin hydroxy-groups of the ligands, and (c) F361(6.51) that interacts with the indole or tetralin ring of the ligands. The occurrence of the latter interaction relies on the establishment of the first two (Figure 2). The involvement of D116(3.32) and S199(5.42) in the interactions with serotonin is consistent with the results of site directed mutagenesis experiments on the 5-HT$_{1A}$ receptor.[29] Moreover, the phenylalanine homologous to F361(6.51) has been suggested to interact with the aromatic ring of epinephrine, based upon the results of biochemical experiments on the α_{1b}-AR.[34] In the selected MD trajectories, the rings of both agonists also perform persistent interaction with Y195(5.38). Alanine substitution for Y5.38 has been found to reduce significantly the affinity of both epinephrine and norepinephrine for the α_{1b}-AR.[35] Furthermore, in the last 500 ps of MD simulation, the hydroxy-group of serotonin is found involved in an additional H-bond with T200(5.43), thereby acting as H-bonding acceptor from S199(5.42) and H-bonding donor to T200(5.43) (Figure 6). Interestingly, the establishment of this double H-bonding connection between the agonist and helix 5 is concurrent with the highest degree of destabilization of both the salt bridges involving the E/DRY arginine (i.e. last 500 ps in Figure 7). This result is consistent with site-directed mutagenesis data, which suggest the involvement of T200(5.43) in serotonin-induced activation of the 5-HT$_{1A}$ receptor.[29]

Despite the structural/dynamics differences between the serotonin− and the (R)-8-OH-DPAT−receptor complexes,

in the second half of simulation, they share the breakage of the R134(3.50)-E340(6.30) interhelical salt bridge found in the empty and antagonist-bound receptor forms (Figures 2, 3, and 6). This structural commonality is concurrent with the reinforcement of the interaction between the ligand and F361(6.51) (Figures 2 and 6). F361(6.51) is the most extracellular member of a cluster of aromatic amino acids in helix 6, including F354(6.44) and W358(6.48). In the majority of the inactive and active forms of the 5-HT_{1A} receptor, F361(6.51) interacts with W358(6.48); the latter, in turn, interacts with F354(6.44), the most cytosolic member of the aromatic cluster. The amino acid pair that separates F361(6.51) from W358(6.48) contains a highly conserved proline, i.e., P360(6.50). This proline introduces a bend in helix 6 in the inactive forms of the 5-HT_{1A} receptor (Figure 3), similarly to rhodopsin structure.[4,36] MD analysis suggests that D116(3.32) and S199(5.42) act as anchor points for both serotonin and (R)-8-OH-DPAT to properly interact with F361(6.51). The establishment of the interactions between the agonist and this phenylalanine induces a change in the orientation and interaction pattern of W358(6.48) and, hence, of F354(6.44) (Figures 3 and 6). These changes are concurrent with a reduction in the bend at P360(6.50), as compared to the inactive receptor forms (Figures 3 and 6). These results agree with the computer simulation- and experiment-based hypothesis that GPCR activation would significantly diminish the kink at P6.50.[45] Thus, a cluster of aromatic amino acids and a proline in helix 6 are suggested to mediate the agonist-induced transfer of the structural modification from the extracellular to the intracellular side. The functional importance of this cluster of aromatic amino acids has been previously suggested by computations and experiments on the D2-dopamine receptor and the α_{1b}-AR.[10,14,46]

Another structural feature shared by the agonist-bound receptor forms, and not by the empty and antagonist-bound forms, is the detachment of helices 2 and 4, following an outward movement of helix 3. This effect is properly marked by the change in the interaction pattern of W161(4.50) that, in the active forms, moves away from helix 2, toward the membrane space. The agonist-induced movements of helix 4 agree with the recent inferences of a computational study on ligand-induced 5-HT_{1A} activation.[22]

The inability of WAY100635 to destabilize the double salt bridge interactions that, in the empty receptor form, involve the E/DRY arginine may be due, at least in part, to its inability to establish the same connections between helices 3, 5, and 6 as those that are made by the agonists. In particular, in the selected antagonist−receptor complex, the phenyl-ring on the piperazine moiety of WAY100635 does not interact with F361(6.51) of the aromatic cluster in helix 6. As a consequence, W358(6.48) retains the H-bond with N392(7.45), while P360(6.50) introduces a bend in helix 6, as found in the empty receptor structures (Figure 3). These features are concurrent with persistent charge reinforced H-bonds between R134(3.50) and both the adjacent aspartate and E340(6.30) (Figures 3 and 5), which confer more strength to both the intrahelical and interhelical salt bridges, as compared to the empty receptor. This feature might correlate, at least in part, with the experiment-based hypothesis that WAY100635 may be an inverse agonist at the 5-HT_{1A} receptor.[37,47]

In summary, the results of this study strengthen the hypothesis that the receptor portions close to the E/DRY/W motif, with prominence to the cytosolic extensions of helices 3 and 6, are particularly susceptible to undergo structural modification in response to agonist binding. This is consistent with the experimental suggestion that GPCR activation involves reciprocal motion of helices 3 and 6.[1,27,48] Despite the differences in the structural/dynamics behavior of serotonin and (R)-8-OH-DPAT, when docked into the 5-HT_{1A} receptor, they both exert a destabilization in the intrahelical and interhelical interactions found in the empty and antagonist-bound receptor forms between the arginine of the E/DRY sequence and both D133(3.49) and E340(6.30) (Figures 2−8). Whether the two anionic amino acids play a role in keeping the 5-HT_{1A} receptor in its inactive state still awaits experimental assessment. For both agonists, the chemical information transfer from the extracellular to the cytosolic domains is mediated by a cluster of aromatic amino acids in helix 6, following the interaction of the ligand with selected amino acids in the extracellular half of the receptor, such as D116(3.32), S199(5.42), Y195(5.38), and F361(6.51). The importance of both D116(3.32) and S199(5.42) for serotonin-induced 5-HT_{1A} receptor activation has been already proved,[29] whereas it would be interesting to investigate the role of the aromatic amino acids, such as Y195(5.38), F361(6.51), W358(6.48), and F354(6.44) as highlighted by molecular simulations.

A significant reduction in the bend at P360(6.50), as compared to the empty and the antagonist-bound receptor forms, is one of the features, shared by the agonist-bound forms, that is related to the breakage of the interhelical salt bridge between the E/DRY arginine and E340(6.30) (Figures 2, 3, and 6). Another structural feature, shared by the agonist-bound receptor forms and not by the empty and antagonist-bound forms, is the detachment of helices 2 and 4, as marked by the movement of W161(4.50) away from helix 2, toward the membrane space. The role of the highly conserved W161-(4.50) and P360(6.50) in the 5-HT_{1A} receptor function awaits to be investigated by site-directed mutagenesis experiments.

The computational strategy employed in this study provides detailed insights into the 5-HT_{1A} amino acids responsible for the transfer of the structural changes from the ligand binding site to the G protein-coupling domains. A more realistic picture of the cytosolic domains of the 5-HT_{1A} receptor awaits the modeling of the third intracellular loop.

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