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ORIGINAL PAPER

Homology modeling of the human 5-HT$_{1A}$, 5-HT$_{2A}$, D1, and D2 receptors: model refinement with molecular dynamics simulations and docking evaluation

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Abstract 5-HT$_{1A}$ serotonin and D1 dopamine receptor agonists have been postulated to be able to improve negative and cognitive impairment symptoms of schizophrenia, while partial agonists and antagonists of the D2 and 5-HT$_{2A}$ receptors have been reported to be effective in reducing positive symptoms. There is therefore a need for well-defined homology models for the design of more selective antipsychotic agents, since no three-dimensional (3D) crystal structures of these receptors are currently available. In this study, homology models were built based on the high-resolution crystal structure of the β_2-adrenergic receptor (2RH1) and further refined via molecular dynamics simulations in a 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) lipid bilayer system with the GROMOS96 53A6 united atom force field. Docking evaluations with representative agonists and antagonists using AutoDock 4.2 revealed binding modes in agreement with experimentally determined site-directed mutagenesis data and significant correlations between the computed and experimental pK_i values. The models are also able to distinguish between antipsychotic agents with different selectivities and binding affinities for the four receptors, as well as to differentiate active compounds from decoys. Hence, these human 5-HT$_{1A}$, 5-HT$_{2A}$, D1 and D2 receptor homology models are capable of predicting the activities of novel ligands, and can be used as 3D templates for antipsychotic drug design and discovery.

Keywords 5-Hydroxytryptamine · Dopamine · Molecular dynamics simulations · Homology modeling · Computational docking

Introduction

Serotonin and dopamine receptors are distributed abundantly in the human central nervous system. They are thought to be involved in neuromodulation, and have been targeted for many neurological brain disorders such as schizophrenia, depression, bipolar disorder, and Parkinson's disease. First-generation typical antipsychotics (which block D2 receptors) and second-generation atypical antipsychotics (which act as partial D2 receptor agonists and 5-HT$_{2A}$ receptor antagonists, e.g., risperidone, ziprasidone and olanzapine) are reported to be effective at reducing positive symptoms but not negative symptoms and cognitive impairment in many treated patients. In recent studies, 5-HT$_{1A}$ or D1 receptor agonists have been postulated to improve cognitive or negative symptoms in schizophrenia patients, although clinical evidence is still lacking [1, 2]. Hence, a compound that acts as both a partial 5-HT$_{1A}$ or D1 receptor agonist and a D2 receptor antagonist may be a promising candidate for a more potent antipsychotic.

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However, the lack of three-dimensional structures of these receptors has hindered the development of such receptor-selective antipsychotics. Fortunately, these receptors share similar core structures with other bioamine G protein-coupled receptors (GPCRs), with seven conserved transmembrane regions being observed. Using the high-resolution 2.40 Å X-ray crystal structure of the human β_2-adrenergic receptor (2RH1), the first human GPCR template to be solved (in 2007) [3], reliable homology models of other bioamine GPCRs can now be produced. In general, a good homology model should be able to reproduce experimental results. However, the model may be inaccurate if the relationship between the model and the template is too distant, especially when the sequence similarities between them are less than 30%. Refining small to medium-sized homology-based protein models using molecular dynamics simulations of tens to hundreds of nanoseconds has been reported to be a useful approach for improving the structures of these models [4]. Such molecular dynamics simulations can be carried out using various types of force field. Although all-atom force fields such as AMBER [5], CHARMM [6], or OPLS-AA [7] are thought to give more accurate results, the use of united-atom force fields such as GROMOS [8] is more computationally economical while it retains accuracy [9, 10]. This is beneficial, especially for long simulations involving large systems, such as a protein embedded in a lipid bilayer.

Homology models of the human serotonin and dopamine receptors based on the 2RH1 structure [11–18] have generally been built with Modeller [19], followed by optimization with either energy minimization or a short run of molecular dynamics simulations (200 ps to 20 ns) with a coarse-grained force field, molecular mechanic force field (MMF94), OPLS2005, or the CHARMM27 force field. These models have then been either qualitatively validated (in comparison to the site-directed mutagenesis data) or quantitatively validated (i.e., via enrichment plots or 3D-QSAR analyses) [15, 17, 18].

In this study, homology models of the human 5-HT_{1A}, 5-HT_{2A}, D1, and D2 receptors were built based on the 2RH1 crystal structure using a nonautomated approach, and the modified parameters from Kukol [20] were used to refine the models through 100 ns of molecular dynamics (MD) simulations in a fully solvated lipid bilayer system with the GROMOS96 53A6 force field. These parameters from Kukol were used to simulate how water molecules penetrate into a lipid bilayer, and were able to reproduce the area per lipid to within an accuracy of 3% compared to the experimental value without the need to assume a constant surface area or include surface pressure [21]. Various approaches were used to validate the models: determining the correlations between the computed and experimental pK_i values, comparing the selectivities of various antipsychotic agents towards the four models, and enrichment studies.

Materials and methods

Homology modeling

The amino acid sequences of the human 5-HT_{1A}, 5-HT_{2A}, D1, and D2 receptors, obtained from the web server UniProtKB (http://www.uniprot.org), were aligned with that of the β_2-adrenergic receptor and those of other biogenic amine class A GPCRs using CLUSTALX [22] and adjusted manually. The homology models were built based on the 2RH1 crystal structure using the Biopolymer module in SYBYL [23] and energy minimized with CHARMM [24] using the adopted basis Newton–Raphson (ABNR) method. Neither the N-terminus nor the C-terminus was modeled, because these regions are not visible in the template [3], and the primary aim of this study was to study the ligand binding sites. However, Cys347 in the C-terminus of the D1 receptor has been reported to be important for maintaining the receptor's conformation when binding antagonists [i.e., SCH23390, (+)-butaclamol, and *cis*-flupentixol], activating adenylyl cyclase, and in agonist-induced desensitization [25], so it was maintained. The intracellular loop 3 (IL3) was also modeled as a replacement for the T4 lysozyme in the template because IL3 joins transmembrane helix 5 (TM5) and transmembrane helix 6 (TM6), which may indirectly restrain the movements of the helices. Furthermore, extracellular loop 2 (EL2) has been found to be important in ligand–receptor interactions [26], and all crystal structures of rhodopsin-like GPCRs—including 2RH1—have been shown to exhibit a conserved disulfide bond between EL2 and transmembrane helix 3 (TM3), which acts like an opening lid to the binding pocket of the receptor. Therefore, the conserved disulfide bond between residues Cys106 in TM3 and Cys191 in EL2 of the template was left unaltered during the modeling.

Refinement of homology models

Simulation system and parameters

The minimized homology models of the human 5-HT_{1A}, 5-HT_{2A}, D1, and D2 receptors were embedded into the pre-equilibrated POPC2 128-lipid bilayer (64 lipids in each leaflet) model obtained from Kukol [20] using the protocol described by Kandt and colleagues [27]. All simulations were performed with the GROMACS simulation package version 4.5.3 [28] under periodic boundary conditions in a rectangular box using the parameters from Kukol [20]; i.e., a constant temperature of 298 K with the Berendsen [29] thermostat and a coupling time constant of 0.1 ps, semi-anisotropic Berendsen pressure coupling involving separate coupling to the z direction (the bilayer normal) and the xy plane with a coupling time constant of 2.0 ps, a cutoff at 1.4 nm for Lennard–Jones interactions, and a real-space

cutoff of 0.9 nm and particle mesh Ewald (PME) [30, 31] for electrostatic interactions. The LINCS [32] and SETTLE [33] algorithms were used to constrain the lipid molecule bonds and the water molecule bonds, respectively. Lipid, protein, and water–chloride–ion molecules were coupled separately to the thermostat. Internal water molecules were also inserted into the binding pocket using the DOWSER program [34]. All systems were first subjected to a maximum of 50,000 steps of energy minimization or a maximum force of 1000 kJ/mol/nm, followed by a 500 ps of heating to 298 K with restraints on protein and lipid atom P8 (the phosphorus atom of the POPC headgroup) under the NPT ensemble, 5 ns of the NPT ensemble with restraints on the protein atoms, and a 100 ns production run without any restraints and a time step of 2 fs. All simulations were carried out on a dual-processor (quad-core Intel Xeon 2.40 GHz) workstation.

Data analysis

To ensure that the systems were well equilibrated before data was collected for analysis, parameters such as the potential energy, system temperature, and pressure were determined. Kinetic energy and total energy, however, were not taken into consideration because Berendsen coupling [29] is not very accurate when determining the kinetic energy due to its weak coupling in nature. The fluidity of the lipid, which has to be maintained throughout the simulation, was determined using some of the important parameters commonly used in lipid simulation analysis: the deuterium order parameter (SCD) and the area per lipid (AL). To ensure that the protein was already well equilibrated before the data were used for analysis, the root mean square deviation (RMSD) was plotted against simulation time, and the time at which the RMSDs of the protein backbone Cα atoms converged was determined. Trajectories were visualized with the Visual Molecular Dynamics (VMD) program [35] and plotted against time with Grace, a two-dimensional (2D) plotting tool.

Docking evaluation

A set of ligands comprising the endogenous ligand and selective agonists and antagonists (Fig. 1) reported in site-directed mutagenesis experiments was downloaded from ZINC [36] and docked into the selected receptor conformations with AutoDock 4.2 [37], using a Lamarckian genetic algorithm [38] with a flexible ligand and a rigid receptor, a population size of 300, 10,000,000 evaluations, and a maximum of 27,000 generations for 100 GA runs. The ligand–receptor binding interactions at the orthosteric binding sites were viewed with LIGPLOT 4.0 [39]. The correlations of the computed pK_i values of correctly bound ligands with pK_i values from site-directed mutagenesis experiments were determined using the Pearson correlation routine in the statistical package SPSS Statistics version 17.0. The receptor conformation that agreed most strongly with the experimental data was determined.

Model validation

The best homology model of each receptor was further validated using PROCHECK [40], Verify3D [41, 42], and ERRAT [43] to ensure that the models were correctly folded (at least the binding pocket in the TM core). In PROCHECK, a Ramachandran plot was created to check whether the model was folded correctly relative to the crystal structure of proteins solved in the PDB database. In Verify3D, a model is compared to its own amino acid sequence using a 3D profile which is computed from atomic coordinates of the correct protein structures. Incorrect segments of a model usually have profile window plots approaching or falling below a score of zero [44], and a Verify3D score below 0.1 indicates a serious problem in the model [45]. On the other hand, ERRAT is used to differentiate correct and incorrect regions of a protein structure by examining the statistics of pairwise atomic interactions between different atoms that are believed to be nonrandomly distributed in relation to each other in the protein.

Enrichment studies

A set of 1000 drug-like ligand decoys was downloaded from Schrödinger [46, 47] for use in enrichment studies. In order to filter out compounds with high chemical similarities to the known active compounds of each of the target receptors, the decoy sets were prepared with MOLPRINT2D [48]; the ligands used in the docking evaluation and a set of inactives from the ChEMBL12 database formed the active and inactive datasets, respectively. The 3D structures of the inactive compounds were downloaded from the ZINC database. Both the known active compounds and the set of filtered decoys were docked to the selected models using a Lamarckian genetic algorithm [38] with a flexible ligand and rigid receptor, a population size of 300, 1,750,000 evaluations, and a maximum of 27,000 generations for 20 GA runs. The docking results were analyzed using a script written in-house that analyzes the docking poses and preferentially ranks ligands bound to amino acid residues that are known to be important for binding, based on experimental site-directed mutagenesis (SDM) and X-ray crystallography data (if available) (Table S1 of the "Electronic supplementary material," ESM) in addition to the conventional AutoDock 4.2 binding energy scores. The receiver operating characteristic (ROC) curve was then plotted [49] and the area under the curve (AUC) was calculated.

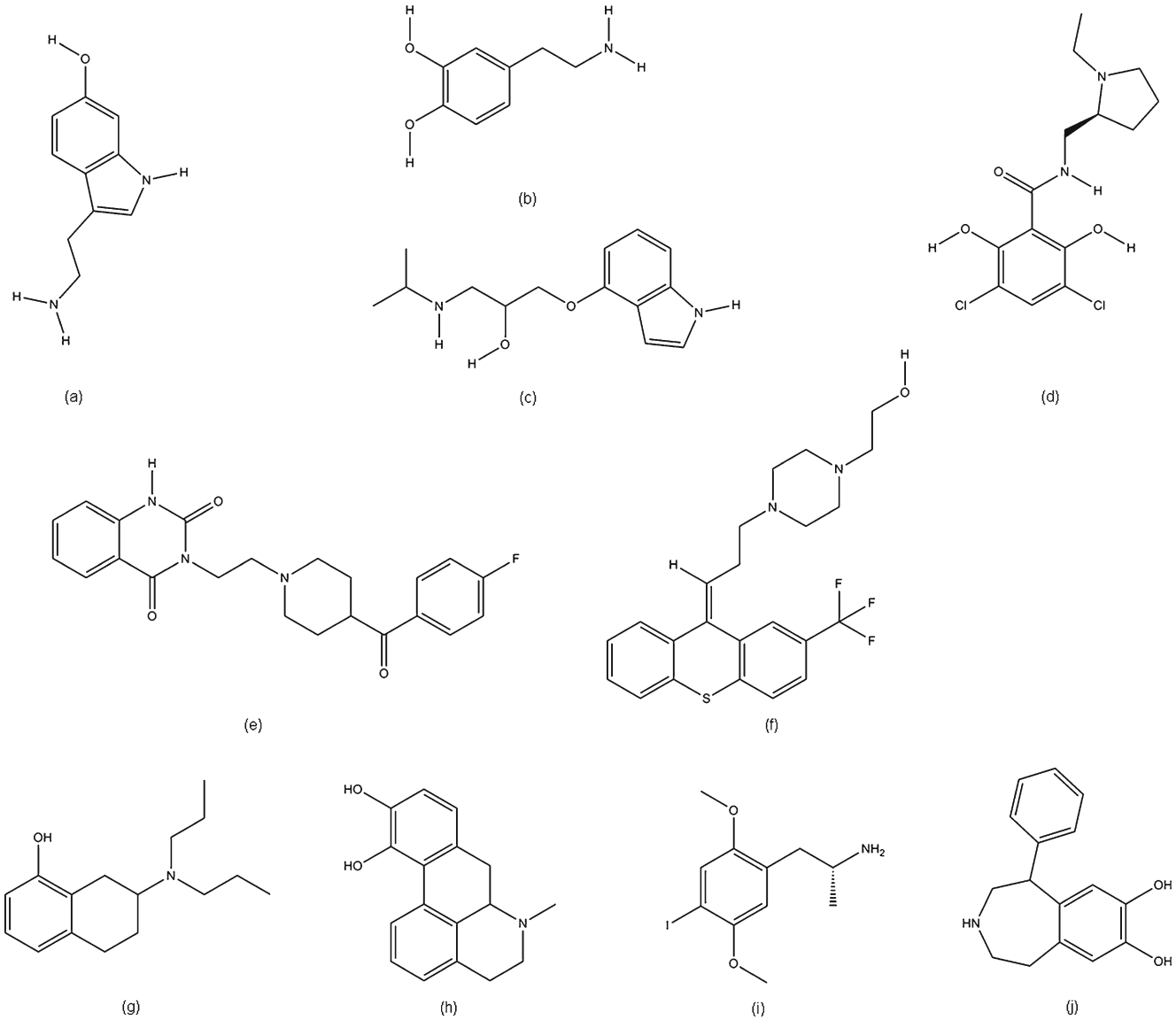

Fig. 1 **a**–**j** Chemical structures of endogenous ligands used: **a** serotonin, **b** dopamine; receptor-selective antagonists: **c** pindolol (5-HT_{1A}), **d** raclopride (D2), **e** ketanserin (5-HT_{2A}), and **f** *cis*-flupentixol (D1); receptor-selective agonists: **g** 8-OH-DPAT (5-HT_{1A}), **h** apomorphine (D2), **i** DOI (5-HT_{2A}), and **j** SKF38393 (D1)

Results and discussion

Refinement of homology models using molecular dynamics simulations with the GROMOS96 53A6 united-atom force field

Based on plots of their potential energy, the 5-HT_{1A}, 5-HT_{2A}, D1, and D2 receptor systems appeared to stabilize after 20 ns, 50 ns, 40 ns, and 60 ns of the production run, respectively. The temperature and pressure of each system also remained stable throughout the simulations (Figs. S1A–S4A of the ESM). For the lipid bilayer system, after taking into account the area occupied by the protein, the average area per lipid, as determined using GridMAT-MD [50], also started to stabilize after 20 ns at a value of approximately 0.57 nm^2 for the 5-HT_{1A} and D1 receptor systems and 0.61 nm^2 for the 5-HT_{2A} receptor systems (Fig. 2). On the other hand, the D2 receptor systems showed a stable average area per lipid throughout the 100 ns of simulations with a value of approximately 0.61 nm^2. This is in agreement with experiments, as the normal measured value of the area per lipid of a pure POPC lipid bilayer has been reported to lie in the range from 0.54 nm^2 to 0.68 nm^2 [51] depending on the temperature at which the experiments are conducted. Nonetheless, the observed values were slightly lower than the value of 0.69 nm^2 reported for pure POPC simulations by Kukol.

There are two possible explanations for this difference. First, the decrease of area per lipid observed may be due to stronger interactions between the hydrophobic peptides and

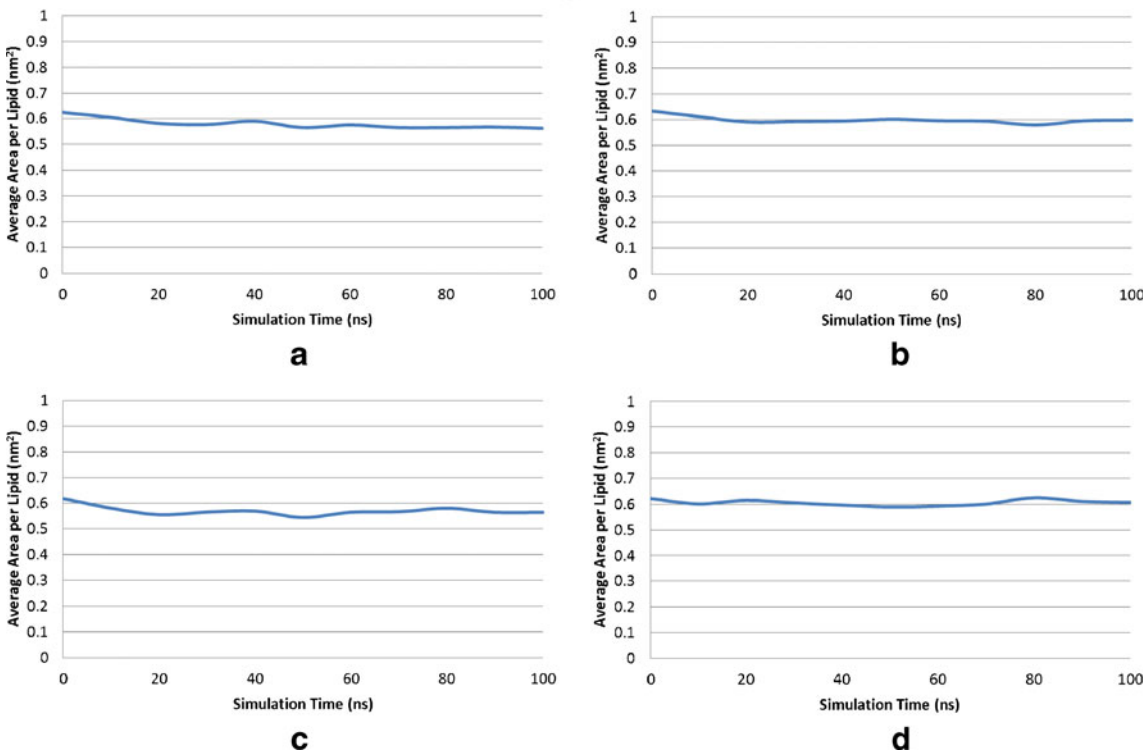

Fig. 2 **a**–**d** Average area per lipid (after subtracting the area occupied by the protein) over 100 ns of a production run in molecular dynamics simulations of the human receptor models in a POPC lipid bilayer: **a** 5-HT_{1A}, **b** 5-HT_{2A}, **c** D1, and **d** D1

the POPC lipids compared to the interactions between the lipids themselves [10]. However, this explanation appears unlikely, as the parameters from Kukol were derived from the GROMOS96 53A6 force field alone, not in combination with other lipid models such as the one developed by Berger et al. [52]. In addition, the deuterium order parameters of the acyl chains (Figs. S1B–S4B of the ESM), were also shown to be in agreement with experiments for frames taken after the time points when the receptor systems started to stabilize. These observations indicate that the lipid bilayer systems did not go into a gel phase and the fluidity of the system was maintained. Consistent shrinking (which is common if a balance is not achieved for the lipid–protein interactions) was also not observed, as the area per lipid stabilized after a few nanoseconds of simulation. Hence, the observed decrease in area per lipid is more likely to be due to the rearrangement of the receptors after positional restraints on the backbone of the proteins were removed in production runs.

The RMSD values for Cα of the 5-HT_{1A}, 5-HT_{2A}, D1, and D2 receptor protein backbones (whole receptors and their transmembrane regions) also displayed relatively stable patterns after 20 ns (5-HT_{1A}), 50 ns (5-HT_{2A}), 40 ns (D1), and 60 ns (D2) compared to the starting structure (Fig. S5 of the ESM), showing that the systems were sufficiently equilibrated. Hence, conformations from the stable trajectories after these time points were extracted and subjected to docking with agonists and antagonists.

Selection of receptor conformations from MD simulation

Clustering methods for selecting receptor conformations for docking studies from within the stable MD trajectories, as provided in the Gromacs utility (i.e., the single linkage, Jarvis–Patrick, Monte Carlo, diagonalization, and GROMOS methods), were unable to give consistent results, with conformations from the same cluster (RMSD cutoff at 0.1 nm) showing different binding properties when the same ligand was docked (results not shown). These observations suggest that a minor change in the binding site may affect the overall binding properties. Although the use of a more stringent RMSD cutoff (e.g., at a value below 0.1 nm) resulted in more accurate clustering results, the number of clusters increased tremendously at the same time, hence defeating the purpose of using a clustering algorithm.

Therefore, conformations from different local minima, together with those around local minima, were selected for docking based on two assumptions: (i) ligands bind to a receptor when it is in a stable conformation (local minimum); (ii) the formation of the ligand–receptor complex gives a stabilizing effect, so conformations around a local

minimum are also potential docking targets. The top 100 lowest-energy conformations from the stable MD trajectories were first docked with the endogenous ligand and then with selective agonists and antagonists (Fig. 1). For conformations that correctly bound the endogenous ligand but not the selective agonists and/or antagonists, 60 conformations around the local minimum conformation were selected for docking with the ligands. Receptor conformations with ligand–receptor interactions that agreed with the site-directed mutagenesis data and with good binding energies were selected for further docking studies.

Correlations between computed pK_i and experimental pK_i values

Based on docking analysis for the 5-HT_{1A}, 5-HT_{2A}, D1, and D2 receptor models, conformations at 72428 ps, 57210 ps, 63922 ps, and 79062 ps, respectively, were identified that gave significant correlations between experimental and computed pK_i values (Table 1 and Fig. 3), with accompanying linear regression (r^2) values in the range 0.7–0.9. The moderate r^2 values observed may be due to the limitation of the AutoDock program used in this study. AutoDock has been reported by previous studies to perform poorly when correlations between docking scores and known bioactivities were evaluated [53], although AutoDock scored reasonably well in terms of yielding a pose in agreement with the crystallographic conformation.

Model validation

PROCHECK

From a PROCHECK [40] analysis of the generated receptor models (Fig. S6 of the ESM), the proportions of the residues in allowed regions (most favored regions, additional allowed regions, and generously allowed regions) were found to be 99.4%, 99.0%, 99.1%, and 99.5% for the 5-HT_{1A}, 5-HT_{2A}, D1, and D2 receptors, respectively, which suggest that the models are reasonably normal [57]. Residues in disallowed regions are all in loop regions. The G factors were also found to have overall averages of −0.67 (5-HT_{1A}), −0.57 (5-HT_{2A}), −0.64 (D1), and −0.63 (D2), suggesting that the generated models were reasonably normal in terms of their stereochemical properties [58].

Verify3D

The results from a Verify3D [42] analysis of the generated models (Fig. S7 of the ESM) showed that the models were all of reasonable quality. The most problematic segments were in the loop region, with scores of below zero. This implied that some parts of the loop regions may not be correctly folded. However, the TM core region (especially the binding pocket) was not affected. Although some parts of the TM regions appeared to have low Verify3D scores, further investigation of the data revealed that this was mainly due to the method used in this program (i.e., every 20 amino acids are grouped into one profile before a 3D–1D score is given to each of the 20 amino acids in the profile [44]), so problems in the loop regions yielded false errors in the TM regions.

ERRAT

ERRAT [43] analysis of the generated models (Fig. S8 of the ESM) produced overall scores of 93.4%, 94.3%, 95.3%, and 90.3% for the 5-HT_{1A}, 5-HT_{2A}, D1, and D2 models, respectively. 3D structures with resolutions of better than 3.0 Å generally produce values above 91%. Hence, it can be concluded that the generated models are of reasonable quality.

Docking evaluation

Human 5-HT_{1A} serotonin receptor

An investigation of the ligand–receptor interactions for the 5-HT_{1A} receptor model (Fig. 4) revealed a significant difference between the residues involved in the binding of serotonin (agonist) and those involved in the binding of pindolol (antagonist), although both were found to bind in the same binding pocket. The amino group of Asn386 was shown to form hydrogen bonds with the hydroxyl group of pindolol, but

Table 1 Correlation between experimental and computed pK_i values for the receptor models

	5-HT_{1A}	5-HT_{2A}	D1	D2
	Guan [54]	Choudhary [55]	Jensen [25]	Mansour [56]
Correlation coefficient, r	0.815*†	0.837*	0.932*	0.890*
Linear regression, r^2	0.664	0.700	0.868	0.792

* Significant correlations between experimental and computed pK_i were observed with $p<0.01$

† One data point (an outlier) was excluded from the calculation

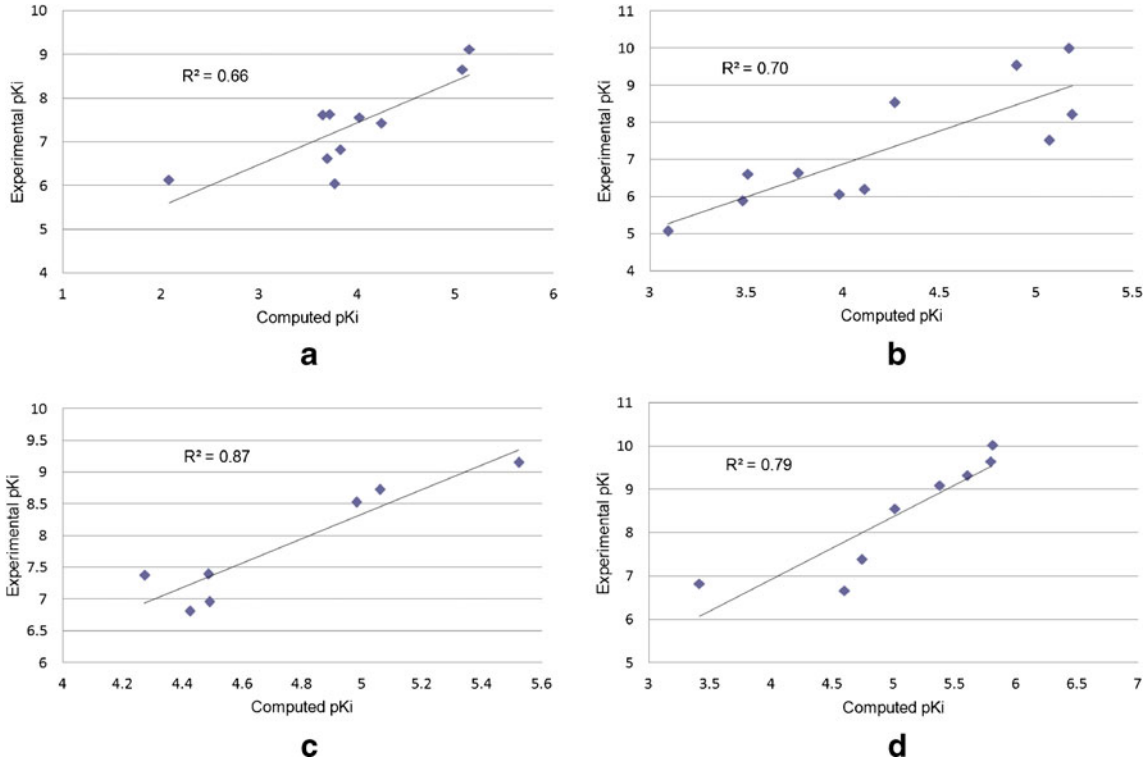

Fig. 3 **a**–**d** Correlation plots of experimental versus computed pK_i values for the receptor models: **a** 5-HT_{1A}, **b** 5-HT_{2A}, **c** D1, and **d** D2

similar interactions were not observed for serotonin. These observations are in agreement with site-directed mutagenesis data, which show that mutating Asn386 to valine (N386V) decreases the affinity of pindolol by 110-fold without significantly affecting the affinity of serotonin [54]. On the other hand, it was also found that when serotonin and pindolol were bound to the receptor, the carboxylate group of Asp116 was involved in hydrogen bonding with the protonated amino groups of both ligands, while the hydroxyl groups of both Ser199 and Thr200 were directly hydrogen bonded with the hydroxyl group of serotonin but not pindolol. These observations are in agreement with a study which reported that mutations of Ser199 and Thr200 result in significantly reduced binding affinities to serotonin but not to pindolol [59]. However, the model did not show a direct interaction of Asp82 with serotonin, as suggested in this report [59]. This phenomenon was also observed in the solved crystal structures of human bioamine GPCRs in complex with agonists [60], inverse agonists [61, 62], partial inverse agonists [3, 63], and antagonists [61, 64, 65] in both active and inactive states. One possible explanation for this observation is that this residue may play an important role in maintaining the conformation of the ligand binding site [66], so a mutation would cause binding to specific ligand(s) to become unfavorable.

Further investigation of the binding pocket in this model revealed that the carboxylate group of Asp82 did indeed form hydrogen bonds with the amino group of Asn396 in TM7, which is known to be conserved among bioamine GPCRs. This has also been observed in the structure of the human β_2-adrenergic receptor (2RH1), where Asp79 was found to bind indirectly to Asn322 in TM7 (corresponding to Asp82 and Asn396 in 5-HT_{1A}, respectively) via a network of hydrogen bonds connecting with three water molecules and the residue Ser318. Mutating Asp82 to asparagine or Asn396 to alanine, valine, or phenylalanine therefore may have prevented the formation of a hydrogen bond between the carboxylate group of Asp82 and the amino group of Asn396, resulting in a significant change in the conformation of the binding site (as the hydrogen bond between Asp82 and Asn396 is the only hydrogen bond that connects TM2 and TM7 directly), and hence decreasing the binding affinities of serotonin and 8-hydroxy-*N*,*N*-dipropyl-2-aminotetralin (8-OH-DPAT), as reported in experiments [59, 67].

Investigations of binding modes involving other ligands—i.e., agonists [5-carboxamidotryptamine (5-CT), lisuride, 8-OH-DPAT], partial agonists (buspirone, ipsapirone), and antagonists (spiperone, propranolol, alprenolol, labetalol, mesulergine, methiothepin, metergoline)—showed that all agonists and antagonists except for 5-CT were able to dock in binding modes, in agreement with the site-directed mutagenesis data, with only the aryloxyalkylamines (pindolol, propranolol, alprenolol) directly forming hydrogen bonds with

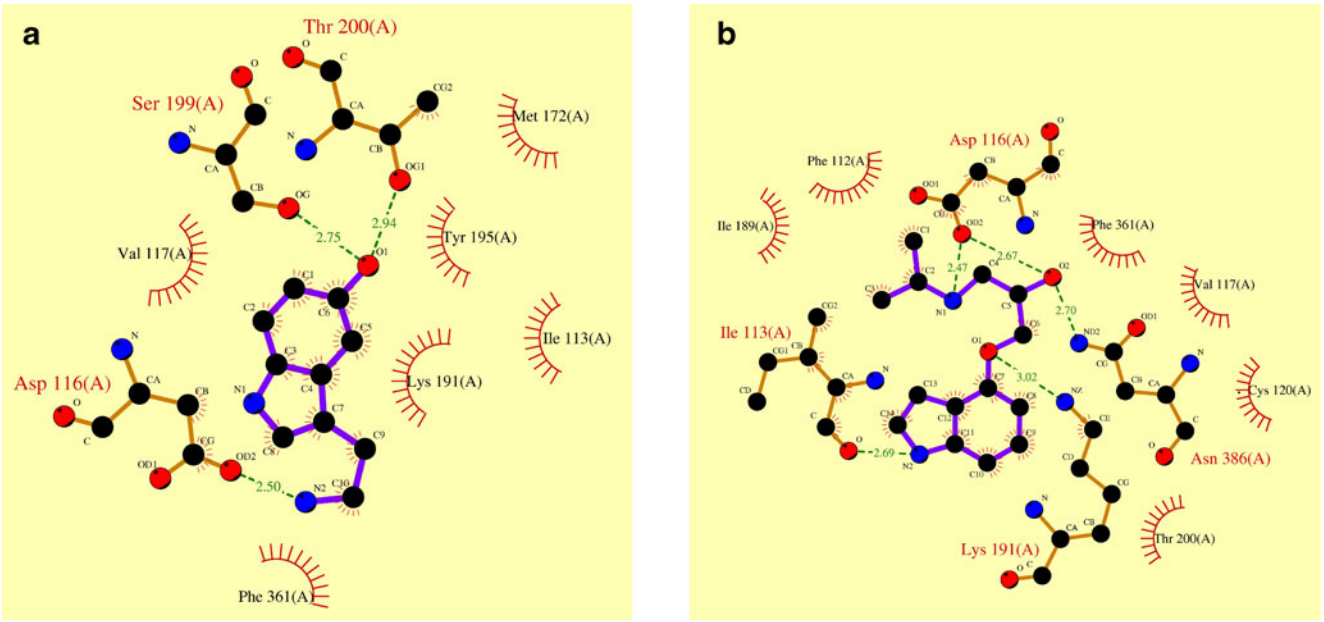

Fig. 4 **a**–**b** Representations of the binding modes of **a** serotonin and **b** pindolol to the human 5-HT_{1A} receptor model, showing the ligands (*purple*), residues involved in hydrogen bonding with the ligand (*brown*) along with their hydrogen bonds (*green*), and residues involved in nonbonded interactions (*red spikes*)

residue Asn386 (Table S2 of the ESM). 5-CT was found to form a hydrogen bond with Asn386 in this model. In apparent contrast with the report, there was little effect on the binding affinity of 5-CT when this residue was mutated to valine [54].

In addition to the above, the model also revealed the formation of hydrogen bonds between Lys191 and pindolol, alprenolol, 8-OH-DPAT, labetalol, mesulergine, metergoline, and 5-CT. This residue has not previously been identified as important for the binding of either agonists or antagonists to the 5-HT_{1A} receptor.

Human 5-HT_{2A} serotonin receptor

An investigation into ligand–receptor interactions for the 5-HT_{2A} receptor model revealed that the model was able to dock both agonists and antagonists, in agreement with site-directed mutagenesis experiments. For instance, when the model was docked with serotonin and ketanserin, the protonated amine group of the ligands was found to form a hydrogen bond with the carboxylate group of amino acid residue Asp155 (Fig. 5). Similarly, most of the other ligands tested were also found to form hydrogen bonds to this residue (Table S3 of the ESM). These results were in agreement with previously reported site-directed mutagenesis experiments on 5-HT_{2A} receptors from rat [68, 69].

In addition to Asp155, Ser159 has also been reported to be important for serotonin binding to 5-HT_{2A} receptors [70]. Mutation of Ser159 to alanine has been observed to result in a significant ~18-fold decrease in serotonin binding affinity and a modest decrease of ~3-fold and ~5-fold in those of *N*,*N*-dimethylserotonin (bufotenine) and lysergic acid diethylamide (LSD), respectively. This difference can be explained by the docking results, which revealed a hydrogen bond between the hydroxyl group of Ser159 and the indole nitrogen atom of serotonin, but not for LSD and bufotenine, although it was proposed in previous studies that Ser159 may be hydrogen bonded to the protonated amine of serotonin, not the indole nitrogen atom [70, 71]. These results also suggest that the modest decrease observed in the binding affinity of LSD and bufotenine upon the mutation of Ser159 was probably due to a decrease in the van der Waals interactions instead of a loss of hydrogen bonding.

On the other hand, the model also showed a hydrogen bond between the hydroxyl group of serotonin and the hydroxyl group of Ser239. This is consistent with the results obtained by Braden and Nichols [72], who found that mutating Ser239 to alanine resulted in a significant decrease in the binding affinity of 5-HT. The study by Braden and Nichols also revealed that mutating Ser242 to alanine only significantly attenuated the binding affinities of LSD, 5-HT and 5-methoxy-dimethyltryptamine (5-MeO-DMT), suggesting that the role of Ser242 in ligand binding is not straightforward. In this study, it was found that Ser242 did not form a direct hydrogen bond to either the hydroxyl group or the indole nitrogen atom of the ligand. Instead, this residue was observed to be involved in van der Waals interactions with the ligand.

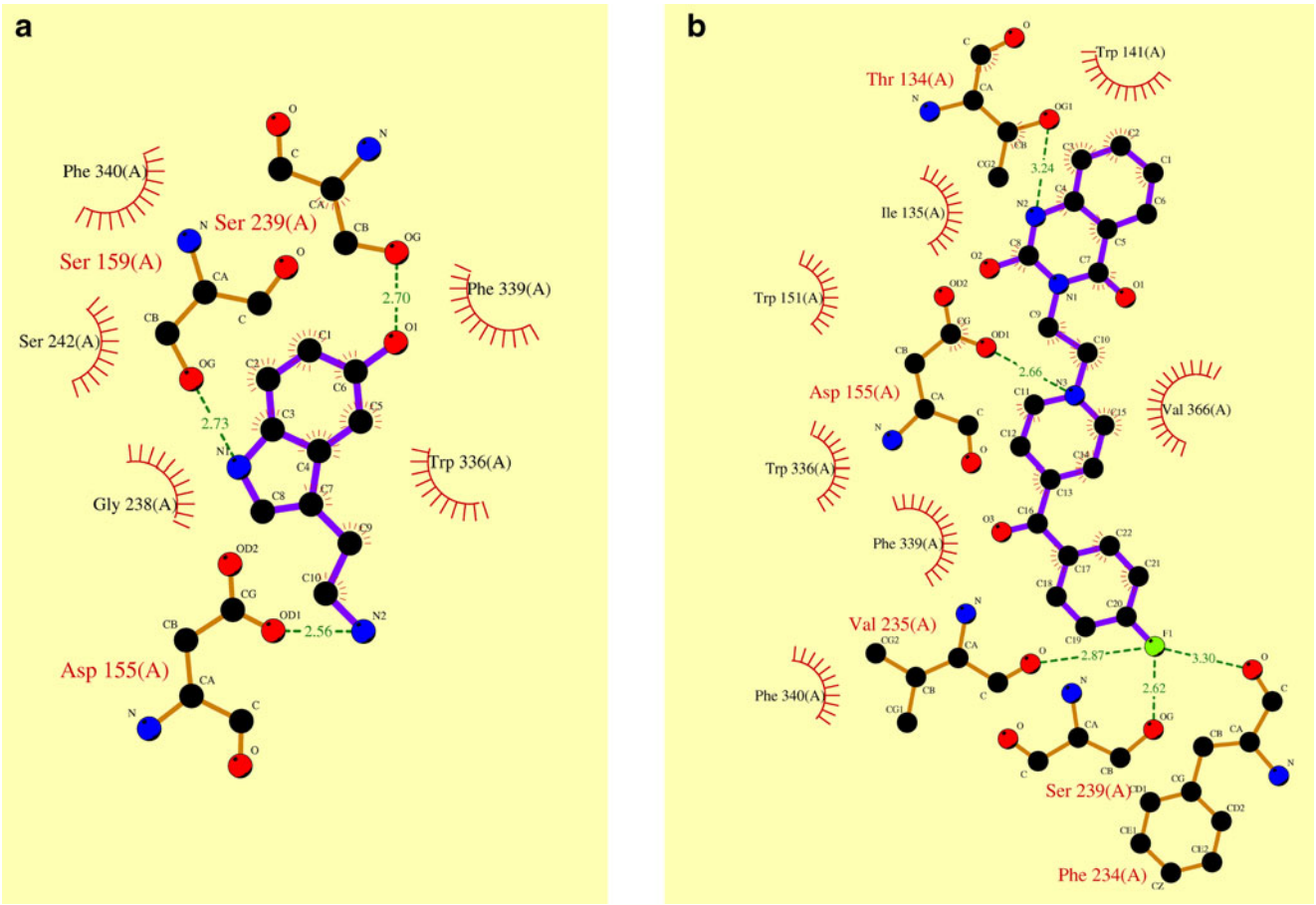

Fig. 5 **a**–**b** Representations of the binding modes of **a** serotonin and **b** ketanserin to the human 5-HT_{2A} receptor model, showing the ligands (*purple*), residues involved in hydrogen bonding with the ligand (*brown*) along with their hydrogen bonds (*green*), and residues involved in nonbonded interactions (*red spikes*)

This work has also shown the importance of the conserved aromatic residues (i.e., Trp336, Phe339, Phe340, and Tyr370) in the binding of both agonists and antagonists. This is consistent with the work of Roth and colleagues, who reported that mutating Trp336, Phe340, and/or Tyr370 to alanine or leucine dramatically altered the binding affinity and/or efficacy of serotonin, DOM, bufotenine, and α-methylserotonin [73]. This may be due to the observed van der Waals interactions between Trp336, Phe340, and/or Tyr370, as well as the network of hydrogen bonds connecting the 4-hydroxylphenyl group of Tyr370 to the ligand via the carboxylate group of Asp155. In another study, Phe340 has also been reported to be crucial to the binding of spiperone, m-CPP, TFMPP, DOI, serotonin, MK-212, bufotenine, and mesulergine, while it has been suggested that Phe339 contributes to the binding of ketanserin [55]. Interestingly, in this study, van der Waals interactions were also observed between these ligands and the residue(s) Phe339 and/or Phe340.

Human D1 dopamine receptor

An investigation into ligand–receptor interactions for the D1 receptor model (Fig. 6) revealed the formation of hydrogen bonds between both the aromatic hydroxyl groups of dopamine and the hydroxyl group of Ser202. Ser198 and Ser199 were also shown to form hydrogen bonds with Ser202, which may have indirectly contributed to the observed binding. These observations are in agreement with site-directed mutagenesis data, which show that mutating both Ser199 and Ser202 results in a 16-fold decrease in the binding affinity of dopamine [74]. Another study has also reported that a single mutation of Ser202 resulted in 50-fold decrease in the binding affinity of dopamine, compared to only a 2-fold decrease for another agonist, SKF38393 [75]. On the other hand, a single mutation of Ser199 resulted in 10-fold and 12-fold decreases in the binding affinities of dopamine and SKF38393, respectively. Ser199 but not Ser202 was also observed to be important in SKF38393 binding for this model, with one of the

hydroxyl groups of SKF38393 forming a hydrogen bond to the hydroxyl group of Ser199, but not to that of Ser202.

It was also observed that the *meta*-hydroxyl group of dopamine forms hydrogen bonds directly with Ile104, indirectly with Cys106 via a network of hydrogen bonds linked to Ile104, and directly with Ser107, all of which are thought to be important in the binding of dopamine based on experimental data [74]. Interestingly, interactions involving the conserved residue in TM2 (Asp70) were observed, but in the second largest cluster and the second lowest energy mode of the dopamine–D1 receptor complex with the hydroxyl group of dopamine bound to the carboxylate group of Asp70 (results not shown). Nonetheless, investigation of the binding mode revealed that an interaction involving both Asp70 and Ser199/Ser202 at the same time (as reported in site-directed mutagenesis [74]) is not possible. This observation suggests that dopamine could bind at two different sites, or this may be due to a similar interaction to that observed and explained in relation to the 5-HT_{1A} model on Asp82.

Investigations of the binding modes of the enantiomeric D1-selective antagonists SCH23388 (*S*) and SCH23390 (*R*) to the model revealed that only SCH23390 (*R*) bound to Ser199 through a hydrogen bond (Table S4 of the ESM). This observation is in agreement with findings by Pollock and colleagues, who reported that a single mutation of Ser199 resulted in a ~70-fold decrease in the binding affinity of SCH23390 (*R*) compared to a ~5-fold decrease in SCH23388 (*S*) [75]. On the other hand, *cis*-flupentixol was observed to interact with Ser199 but not Ser202 (Fig. 5). Similar observations were noted by Pollock and colleagues, who reported that the mutation of Ser199 resulted in a 9-fold decrease in the binding affinity of *cis*-flupentixol [75].

Investigations of binding modes involving other antagonists (butaclamol, bulbocapnine, chlorpromazine, clozapine, eticlopride, flupenthixol, SKF82958, spiperone) showed that all of these ligands except for clozapine and eticlopride formed hydrogen bonds to Asp103 and/or Ser199. These results show that these residues are important in both agonist and antagonist binding to D1 receptors.

Human D2 dopamine receptor

An investigation into ligand–receptor interactions for the D2 receptor model (Fig. 7) revealed the formation of a hydrogen bond between the *meta*-hydroxyl group of dopamine and the hydroxyl group of Ser193, and another between the *para*-hydroxyl group of dopamine and the hydroxyl group of Ser197. Similar interactions were also observed when other agonists were docked [i.e., apomorphine, quinpirole (LY171555), and NPA], but these interactions were not observed when any of the antagonists were docked (Table S5 of the ESM). These observations were in agreement with site-directed mutagenesis experiments, which showed a significant decrease in the binding affinities of agonists when Ser197 was mutated [56]. Similarly, another study by Woodward and colleagues also found that mutating Ser193, Ser194, and/or Ser197 to alanine has little effect on the binding of most antagonists, while all three serine residues participate in the binding of agonists [76].

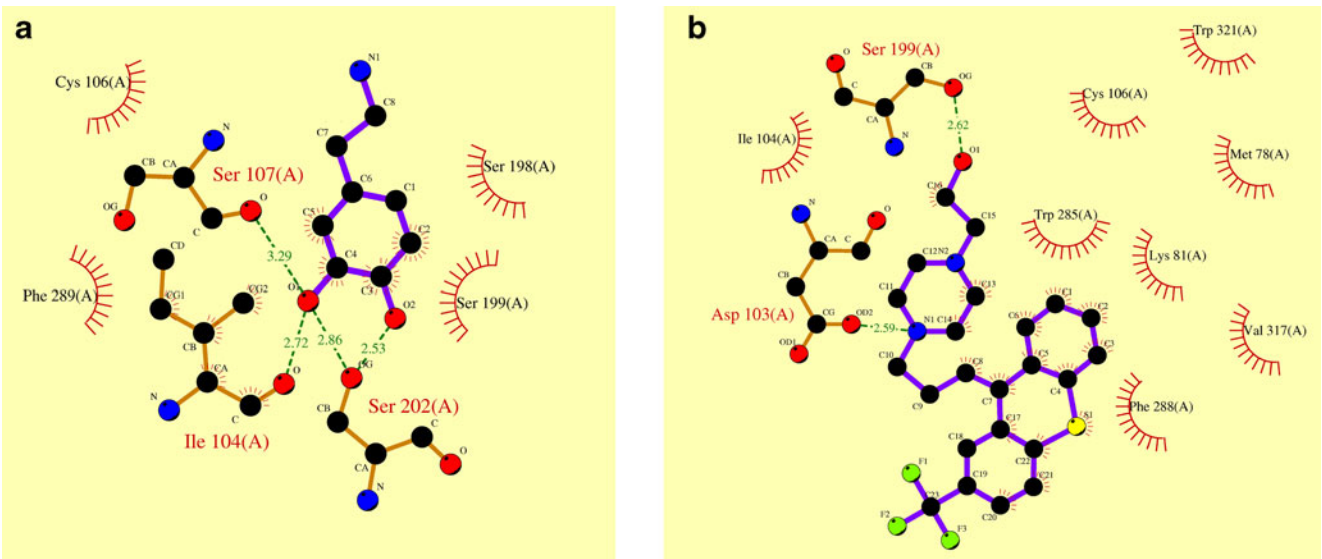

Fig. 6 **a**–**b** Representations of the binding modes of **a** dopamine and **b** *cis*-flupentixol to the human D1 receptor model, showing the ligands (*purple*), residues involved in hydrogen bonding with the ligand (*brown*) along with their hydrogen bonds (*green*), and residues involved in nonbonded interactions (*red spikes*)

The model also displayed a strong hydrogen-bond interaction between the protonated amino group of the ligand and the carboxylate group of the conserved Asp114 when most of the agonists and antagonists were docked. These observations were once again in agreement with experiments by Mansour and colleagues, who reported a significant decrease in the binding affinities of N0437 (agonist) and raclopride (antagonist) upon the mutation of Asp114 [56].

The model also showed that Cys118 was involved in van der Waals interactions with most of the ligands, as this residue was found to be exposed to the binding site crevice and was hydrogen bonded to Asp114. This is in agreement with the work of Javitch and colleagues [77]. In addition, the mutation of Cys118 to a residue with a longer side chain was also reported to cause interference during ligand binding [78]. The results of the analysis also revealed key hydrophobic interactions of Phe389 and/or Phe390 in TM6 with all of the agonists and antagonists docked (Table S4 of the ESM). These residues were shown to be crucial to ligand binding—mutating these residues to alanine resulted in the disruption of binding to several agonists and antagonists, and impaired inhibition of adenyl cyclase activity [79].

Nonetheless, this model did not show a direct interaction of Asp80 with dopamine, as suggested in the work by Neve and colleagues, who reported a decrease in the affinities of both agonists and substituted benzamide antagonists when mutated [80]. As discussed in relation to the 5-HT_{1A} model, Asp80 plays an important role in maintaining the conformation of the binding pocket for binding, rather than being directly involved in the ligand–receptor interactions. This is further supported by the fact that the carbonyl group of Asp80 in TM2 is bound to the conserved residues in TM7 (e.g., Ser419). Therefore, mutating Asp80 to other residues such as alanine (Ala) or glutamic acid (Glu) prevents such interactions from occurring, resulting in changes to the binding pocket conformation.

Investigations into binding modes involving other ligands revealed that all but one of the ligands bind at the binding site with the correct binding mode at the lowest energy and/or largest cluster. The only exception was bromocriptine, which did not form a hydrogen bond with Ser197 as suggested by mutagenesis data [56].

Selectivities of various ligands towards the four receptor models

In this study, the capacity of the models to distinguish the selectivities of various ligands based on their predicted K_i values was also investigated. A total of six antipsychotic agents with different selectivities towards the four human receptors 5-HT_{1A}, 5-HT_{2A}, D1, and D2 were investigated:

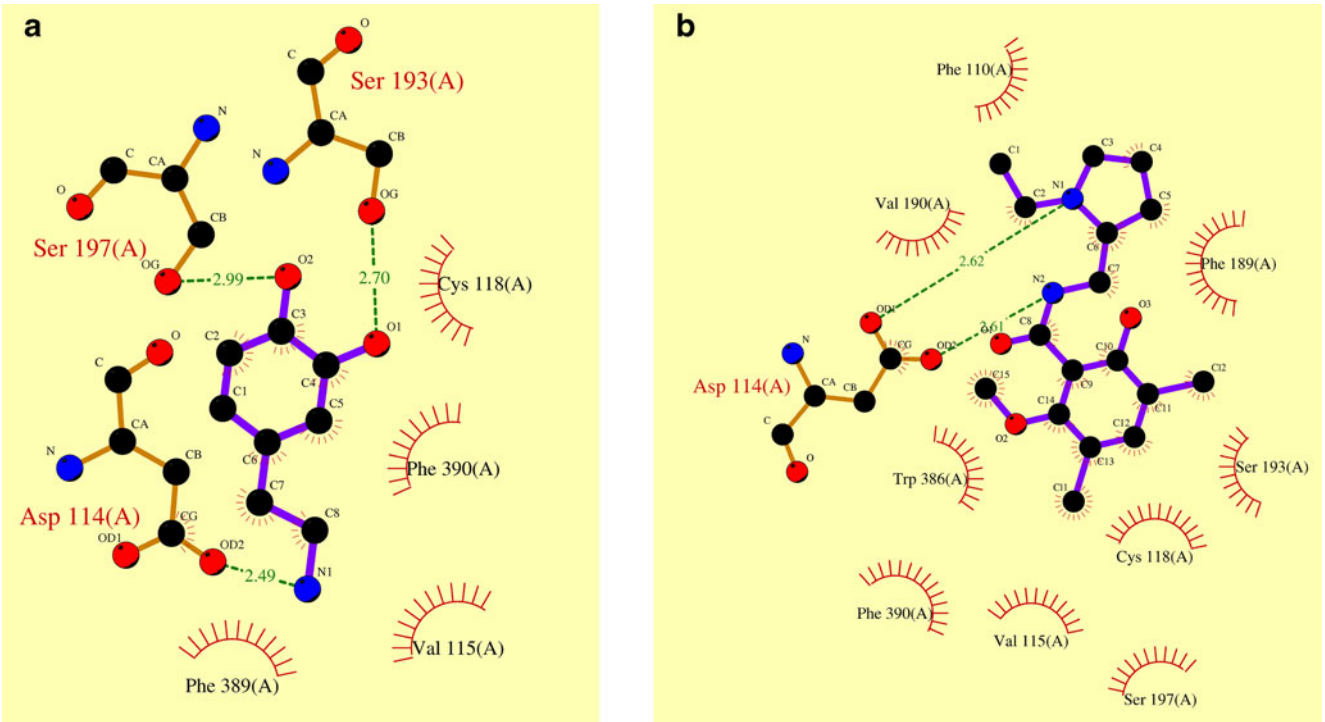

Fig. 7 **a**–**b** Representations of the binding modes of **a** dopamine and **b** raclopride to the human D2 receptor model, showing the ligands (*purple*), residues involved in hydrogen bonding with the ligand (*brown*) along with their hydrogen bonds (*green*), and residues involved in nonbonded interactions (*red spikes*)

the typical antipsychotics haloperidol and clozapine and the atypical antipsychotics iloperidone, risperidone, quetiapine, and olanzapine (Fig. 8). The results are provided in Table 2.

Iloperidone and risperidone were shown to have higher affinities to the 5-HT_{2A} and D2 receptors than to the 5-HT_{1A} and D1 receptors. On the other hand, haloperidol was shown to bind strongly to the D2 receptor, while quetiapine did not show significant selectivity towards any of the four receptors, and it presented its lowest affinity for the D1 receptor. These results were in agreement with the experiments by Kongsamut and colleague [81]. When the predicted K_i values of the drugs were compared to the predicted K_i values obtained for the endogenous ligand (i.e., serotonin for 5-HT_{1A} and 5-HT_{2A}, and dopamine for the D1 and D2 receptor models; Tables S2–S6 of the ESM), it was found that only clozapine showed a higher K_i value in the 5-HT_{1A} model compared to the endogenous ligand, suggesting that clozapine has a reasonably weak affinity for the 5-HT_{1A} receptor. On the other hand, other antipsychotic agents investigated were found to have better affinities for all of the receptors than the endogenous ligands. These results suggest that all of the antipsychotic agents investigated are antagonists with different selectivities for the 5-HT_{1A}, 5-HT_{2A}, D1, and D2 receptors, except for clozapine, which may act as a 5-HT_{1A} receptor agonist. A study by Newman-Tancredi and colleagues confirmed the role of clozapine as a partial agonist [82].

Further investigation of the docking properties revealed that aromatic residues played a significant role in 5-HT_{2A} and D2 receptor antagonism. For instance, haloperidol bound with higher affinity to the human D2 receptor model than the other receptors because there are more aromatic residues in the binding site crevice that are exposed to haloperidol (i.e., Phe110, Phe189, Trp386, Phe389, and Phe390). Similarly, risperidone and iloperidone were also found to bind more strongly to the human 5-HT_{2A} and D2 receptors than to the 5-HT_{1A} and D1 receptors due to the role of the aromatic residues Trp151, Phe234, Trp336, Phe339, and/or Phe340 in the case of the 5-HT_{2A} receptor, and Phe110, Phe189, Trp386, Phe389, Phe390, Tyr408, and/or Trp413 in the case of the D2 receptor. On the other hand, fewer aromatic residues were

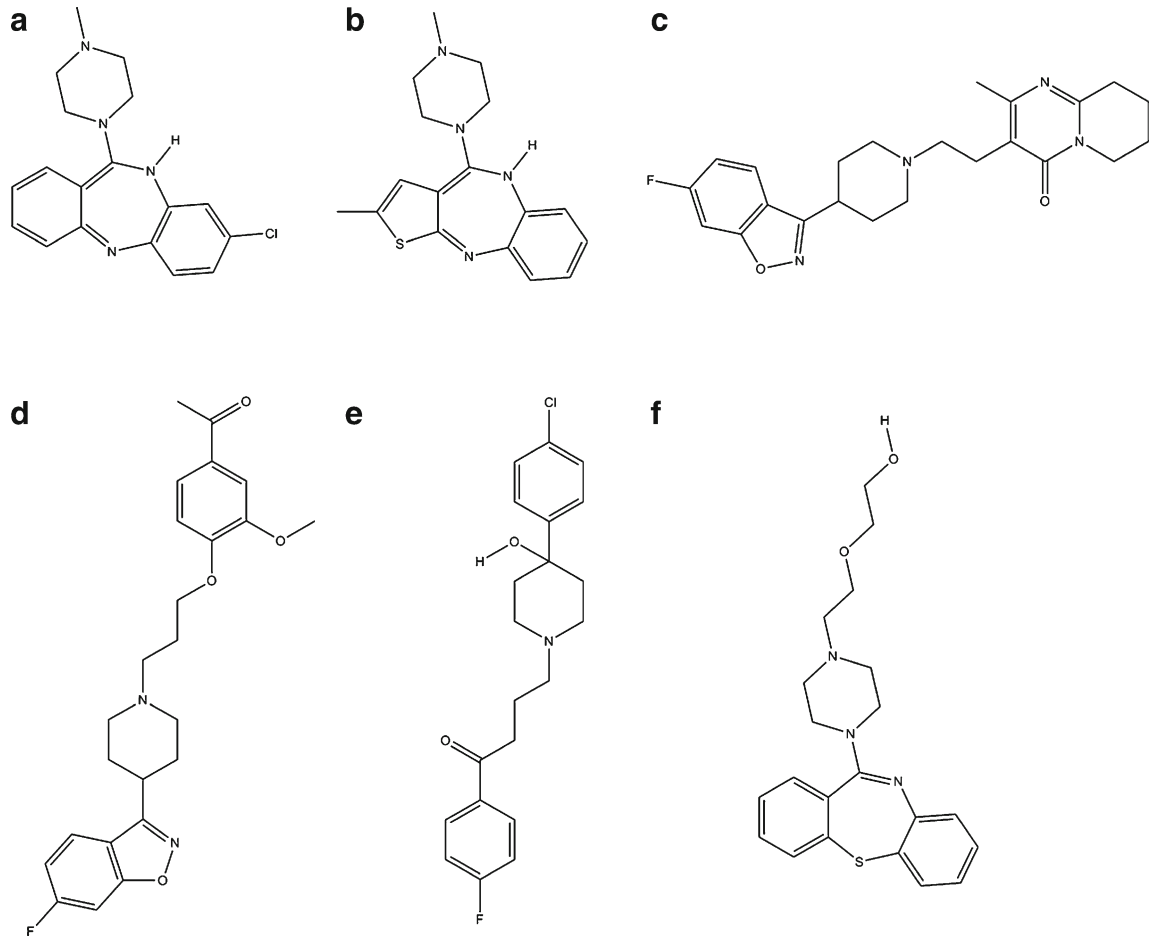

Fig. 8 **a**–**f** Chemical structures of the antipsychotic agents docked into the four receptor models: **a** clozapine, **b** olanzapine, **c** risperidone, **d** iloperidone, **e** haloperidol, and **f** quetiapine

Table 2 The binding affinities of antipsychotic agents to the human 5-HT_{1A}, 5-HT_{2A}, D1, and D2 receptor models. The *highlighted values* represent the trend in selectivities observed across the four models for each antipsychotic agent investigated, indicating significant maxima (*bold values*) or minima (*italicized values*)

	Computed K_i (μM)					
	Iloperidone	Risperidone	Haloperidol	Quetiapine	Clozapine	Olanzapine
5-HT_{1A}	17.87	61.38	**161.15**	75.14	**1510**	**491.93**
5-HT_{2A}	*1.81*	*1.32*	8.53	45.54	48.01	95.17
D1	4.69	2.36	3.77	**97.31**	53.35	101.89
D2	*0.10199*	*0.23415*	*0.67015*	9.84	18.1	6.73
	Experimental K_i (nM)*					
	Iloperidone	Risperidone	Haloperidol	Quetiapine	Clozapine	Olanzapine
5-HT_{1A}	168	570	**>2000**	720	**640**	**4546**
5-HT_{2A}	*5.6*	*1.1*	186	636	23.2	24.2
D1	216	523	82	**1277**	196	35
D2	*6.3*	*2.7*	*2.3*	706	291	37.7

*Values are from Kongsamut et al. [81], with 5-HT1A values for rat from Corbett et al. [83]

observed in the binding site crevices of the 5-HT_{1A} and D1 models. These observations suggest that aromatic residues play a significant role in 5-HT_{2A} and D2 antagonism. In addition, the low binding affinity for 5-HT_{1A} demonstrated by clozapine, olanzapine, and haloperidol may also be partly due to the failure of these ligands to form hydrogen bonds to the conserved residue Asp116 in the 5-HT_{1A} model, resulting in improper orientation. Olanzapine, although very similar to clozapine, showed binding to the conserved residue Asn386 instead. These results clearly show that the ligand–receptor interactions are not as straightforward as originally presumed, so it is very useful to have 3D models of these receptors to guide the design of novel antipsychotic agents.

Enrichment studies

Enrichment studies involving the virtual screening of a set of known active compounds and decoys are widely used to determine the capabilities of homology models. However, the outcome can be misleading if the docking algorithm itself is imperfect. As discussed earlier, although AutoDock performs well in yielding correct binding poses, its scoring function is not so impressive [53]. This was also found to be the case in this study for the docking of the known active compounds and decoys into the 2RH1 crystal structure. As illustrated in Fig. 9a, the ROC enrichment plot obtained when the compounds were ranked based on AutoDock binding energy scores alone (yellow line) is very close to the plot that would be obtained from a random search (red line), with an area under the curve (AUC) value of only 0.62.

In contrast, when a script written in-house, which gave preferential ranking to ligands bound to residues that are known to be important in binding (based on experimental data), was applied in addition to the AutoDock binding energy scores, a clear improvement in the ROC enrichment plot was observed (blue line), and the AUC value increased to 0.78. Hence, the new scoring procedure was applied to the generated models (Fig. 9b–e), and they were found to yield AUC values of 0.84, 0.78, 0.79, and 0.86 for the 5-HT_{1A}, 5-HT_{2A}, D1, and D2 models, respectively. The fact that these values are all in excess of 0.70 indicates that the models are able to differentiate between the active compounds from the decoys moderately accurately [84].

Conclusions

Homology models of the human 5-HT_{1A}, 5-HT_{2A}, D1, and D2 receptors, generated from a high-resolution crystal structure of the β_2-adrenergic receptor, have been refined with molecular dynamics simulations in a solvated lipid bilayer using the GROMOS96 53A6 force field with modified parameters. An evaluation of the docking of these models with representative agonists and antagonists using AutoDock 4.2 revealed binding modes in agreement with site-directed mutagenesis experiments. Some novel binding interactions were also observed, which may be useful for future site-directed mutagenesis studies. Furthermore, the models gave moderate-to-strong correlations between computed and experimental pK_i values, and are able to distinguish between antipsychotic agents with different selectivities and binding affinities for the four receptors. In addition, the models were able to differentiate active compounds from decoys, and are thus capable of predicting the activities of novel ligands. Therefore, it can be concluded that the generated

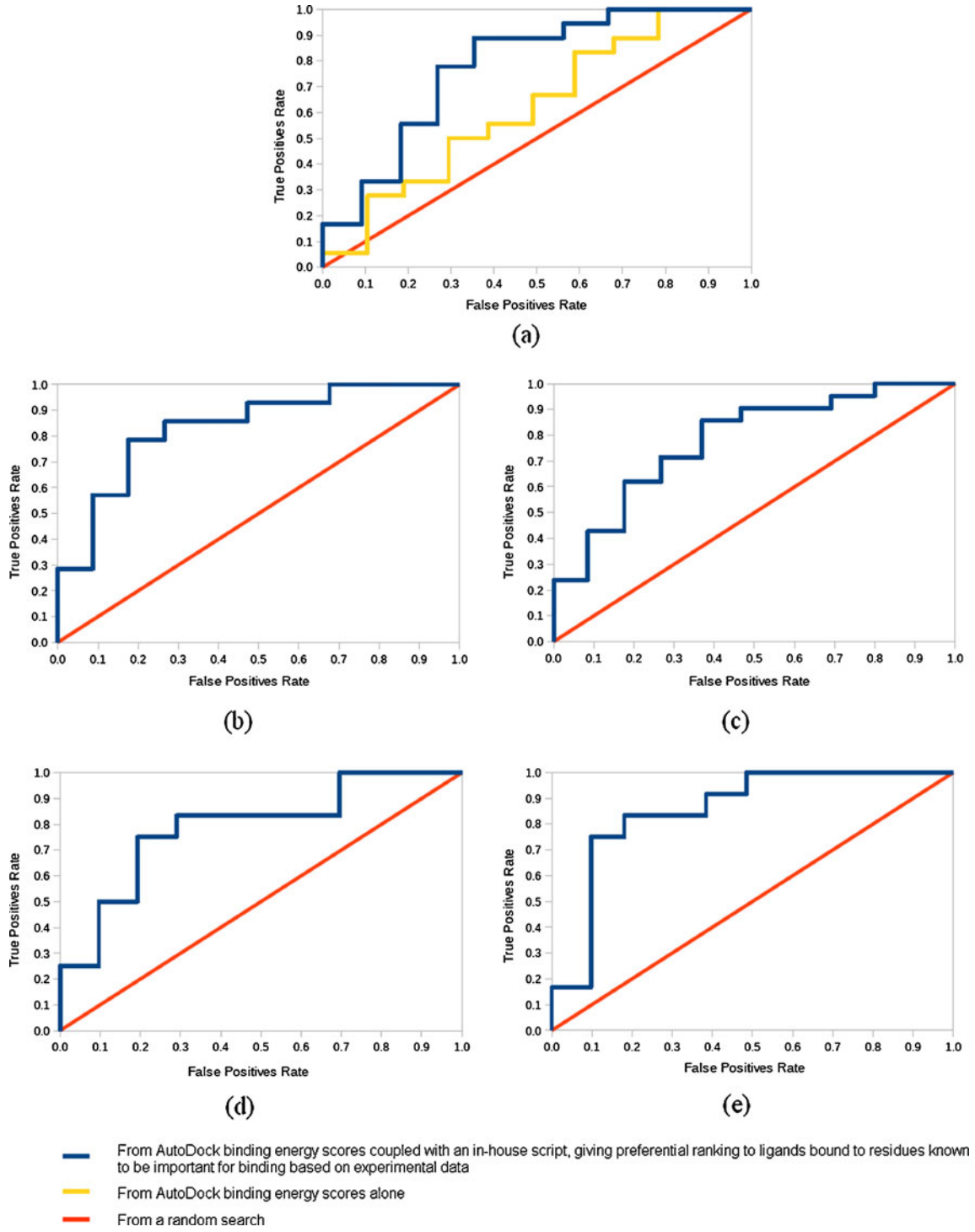

Fig. 9 **a**–**e** ROC enrichment plots for the receptor models: **a** β_2-adrenergic (2RH1), **b** 5-HT_{1A}, **c** 5-HT_{2A}, **d** D1, and **e** D2

homology models of the human 5-HT_{1A}, 5-HT_{2A}, D1, and D2 receptors can be used as good 3D templates for antipsychotic drug design and discovery.

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