Abstract

Dopamine (DA) receptors, a class of G-protein coupled receptors (GPCRs), have been targeted for drug development for the treatment of neurological, psychiatric and ocular disorders. The lack of structural information about GPCRs and their ligand complexes has prompted the development of homology models of these proteins aimed at structure-based drug design. Crystal structure of human dopamine D3 (hD3) receptor has been recently solved. Based on the hD3 receptor crystal structure we generated dopamine D2 and D3 receptor models and refined them with molecular dynamics (MD) protocol. Refined structures, obtained from the MD simulations in membrane environment, were subsequently used in molecular docking studies in order to investigate potential sites of interaction. The structure of hD2 and hD2L receptors was differentiated by means of MD simulations and D2 selective ligands were discriminated, in terms of binding energy, by docking calculation. Robust correlation of computed and experimental Kᵢ was obtained for hD2 and hD2 receptor ligands. In conclusion, the present computational approach seems suitable to build and refine structure models of homologous dopamine receptors that may be of value for structure-based drug discovery of selective dopaminergic ligands.

Introduction

The dopaminergic systems in the central nervous system (CNS) have been extensively studied over the past 50 years [1]. Dopamine exerts its action through five distinct G-protein coupled receptors (D₁–5 receptors), grouped in two classes, D₁-like and D₂-like receptors, that differ in their signal transduction, binding profile and physiological effects [1]. D₁-like receptors (D₁ and D₅) are principally coupled to stimulatory Gₛ-proteins and enhance the activity of adenylyl cyclase (AC), whereas D₂-like receptors (D₂, D₃, and D₄) are primarily coupled to inhibitory Gᵢ-proteins and suppress the activity of AC [1].

Alternative splicing of D₂ receptor mRNA leads to generation of two isoforms: D₂ short (D₂S) and D₂ long (D₂L), which have been associated (though not exclusively) with presynaptic and postsynaptic populations of D₂ receptors, respectively [2]. The difference between these two splicing isoforms is represented by 29 amino acid residues in the III intracellular loop (3ICL), involved in the G protein coupling. The D₂S is mainly considered differentiated by means of MD simulations and D₂ selective ligands were discriminated, in terms of binding energy, by docking calculation. Robust correlation of computed and experimental Kᵢ was obtained for hD2 and hD2 receptor ligands. In conclusion, the present computational approach seems suitable to build and refine structure models of homologous dopamine receptors that may be of value for structure-based drug discovery of selective dopaminergic ligands.
design studies were carried out successfully by Lopez et al. [28] who reported benzolactam derivatives with distinct selectivity against D₃ and D₂ receptors; functionalized benzolactam compounds were reported to have D₂ dopaminergic agonism [29]. Recently, Tscharmenter et al. [30] synthesized heterocyclic dopamine surrogates, among which one compound (biphenylcarboxamide (S)-5a) has a very high affinity (27 pM) at the D₃ receptor and high selectivity over D₂ subtype.

The crystal structure of hD₃ has been solved [31] and identified as a powerful tool for structure-based drug discovery of selective dopaminergic D₂-like ligands [32]. This crystallized receptor is a hD₃-lysozyme chimera, where the 3ICL is replaced by the lysozyme protein; moreover, the receptor bears the mutation Leu197Thr in order to increase the thermal stability of the system. Recently, the determination of the crystal structure of hD₃ receptor and subsequent efforts in molecular modeling led to successful prediction of the pose of eticlopride in complex with a refined homology model of D₃ receptor [33]. Kartagere et al. [34] analyzed in 2011 the binding mode of preferential D₃ ligands of homology models of hD₃ and hD₂ receptors using the hD₃ receptor.

Equilibration steps and simulations were carried out using NAMD v1.8.7 [38]. Before MD simulations the systems were equilibrated as follows: i) MD of lipid tails for 50 ps (time-step = 1 fs) while protein, water, ions and lipid head groups were kept fixed; ii) equilibration for 100 ps (time-step = 1 fs) of water-Ions-lipids, while proteins were kept fixed by applying harmonic constraints; iii) 500 ps (time step = 1 fs) of system equilibration, with no constraints applied to molecules. After the described steps of equilibration, 3 ns of MD simulation were carried out with time-step of 2 fs, collecting trajectory data every 10 ps. The SHAKE algorithm, which constrains the hydrogen-heavy atom bonds, was applied. Equilibration steps and simulations were carried out using NAMD v2.7 [39]. Langevin dynamics and piston were used to maintain constant temperature (300 K) and pressure (1 atm) during simulation. The area per lipid was maintained constant, after the equilibration steps (NPAT ensemble). The particle number of systems was 83242 for hD₃-lipids-water-Ions and 83429 for hD₂ in membrane. Periodic Boundary Conditions (PBC) and Particle Mesh Ewalds (PME) method [40] were used to treat long-term electrostatics (time-step of 4 fs). The cut-off at 10 Å was applied to Van der Waals and coulombic interactions and switching functions started at 9 Å. First stage minimization was performed using the steepest descent algorithm whereas the conjugate gradient was used during production runs.

Docking and Virtual Screening

We carried out two different molecular docking studies using Vina and AD4.2 software. Vina [41] is an accurate algorithm faster than AD4.2; for this reason it was used for docking calculation of a large group of D₂-like ligands and for virtual screening study. AD 4.2 [42] provided the best prediction of pose of eticlopride in the hD₃ homology model, thus we have chosen it for accurate docking calculation such as prediction of Kᵢ of well-known D₂-like agonists docked into the refined homology models of hD₃ and hD₂ receptors. File preparation for AD4.2 docking calculations was carried out using the AutodockTool (ADT), a free graphics user interface (GUI) of MGL-tools.

The search space for all docking calculations included the orthosteric binding pocket individuated by eticlopride in 3PBL, the allosteric binding pocket reported by Chien et al. [31] and the extracellular domain of receptors. An high exhaustiveness, 32, was used in Vina calculation because the search space applied to hD₃ and hD₂ receptors is relatively wide. In calculations carried out in
with AD4.2 we chose, as search algorithm, the time-consuming Lamarkian genetic algorithm (GA), that generated the best docking results for eticlopride in hD3 homology model. Hundred iterations of GA with 2,500,000 energy evaluations per run were carried out. Population size was set to 150 and a maximum of 27,000 generations per run was carried out, followed by automatic clusterization of poses. Top scored (lowest energy) and more populated poses with orthosteric binding, as reported by Kortagere et al [34], were selected for analysis of ligand-protein interactions using the GUI ADT. AD 4.2 uses a semi-empirical free energy function and a charge-based method for desolvation contributes; the free energy function was calibrated using a set of 188 structurally known ligand-complexes with experimentally determined binding constants [43]. The binding energy of ligand poses (Kcal/mol) is the sum of intermolecular energy, internal energy of the ligand and torsional free energy minus the unbound-system energy (see in Supporting Information S1 about the calculation of K, from AD4.2 binding energy values and Supporting Information S2 for ligand poses and optimized structure of receptors).

**Ligand Dataset**

Structure files of ligands were retrieved from PubChem [44], ZINC database [45], and, when not available there, from PRODRG web service (http://davapc1.bioch.dundee.ac.uk/prodrg/), as.mol2 files [46]. Whenever a conversion of file format was necessary it was done by Open Babel [47]. Protonation state of ligands was assigned at pH = 7.4. Three replicas of dockings were carried before and after MD simulations in order to assess the structure differentiation of homology model simulated in membrane. The following ligands were used in fast docking calculations with Vina: r-7-OH-DPAT, r-7-OH-IPAP, r-7-OH-PPPP, r-7-OH-PBZI, r-7-OH-PAM, bromocriptine, lergotrile, lisuride, pergolide, cianegoline, cabergoline, SDZ-GLC-756, PD128907, pramipexole, rotigotine, ropinirole, eticlopride, U99194A, Ru24213, GR103691, r-GSK89472, s-GSK89472, s-naftadotride, NGB2904, PG10137, PNU177864, SB-269-652, S33084, SB277011A, S14297, S17777 and compounds of the USC series from Ortega et al [29] (USC-A401, USC-B401, USC-H401, USC-I401, USC-K401, USC-M401). The D3 agonists, represented in Figure 1, r-7-OH-DPAT, r-7-OH-IPAP, pramipexole, ropinirole, rotigotine, quiniprine, dopamine, PD128907 and cis-8-OH-PBZI (cis-8-hydroxy-3-(n-propyl)-1H-benz[e]indole) were docked with AD4.2 into the hD3 and hD2L receptors optimized by MD; the predicted K, values were correlated to the experimental ones. Eighty nine compounds, retrieved from ZINC database, were used to build a small focused drug-like database of ligands (according to the Lipinski’s rule of five and similar at 70% to pramipexole); they were docked with Vina into hD3 and hD2L refined receptors. Structural alignments of proteins and figures were done with the molecular visualization software OpenPyMOL. All software utilized in our study were open source or under free of charge academic license. Computational hours were provided by the GRID service “Consorzio Cometa” [http://www.consorzio-cometa.it/].

**Results**

**Homology Modeling**

We built the homology models of hD3 and hD2L receptors. Two disulfide bridges were modeled in hD3 receptor according to the crystal structure 3PBL [31], the canonical one that connect the 2ECL with the III helix and the disulfide bridge in the 3ECL involving residues Cys 355 and Cys 358. In hD2L receptor only the conserved disulfide bridge was modeled, because we considered that a single residue of distance between the two conserved cysteine residues (Cys 399 and Cys 401) may lead to unstable disulfide bond. Validation for the hD3 model, by docking eticlopride with Vina and AD4.2 was performed. Both software were able to reproduce the eticlopride conformation in the binding pocket; AD4.2 gave the lowest root mean square deviation (RMSD, 0.6 Å) and better reproduced the internal H-bonds (Figure 2A), compared to VINA (Figure 2B), that gave 0.6 Å RMSD for re-docked eticlopride. We have evaluated the similarity of hD3 and hD2L homology models by means of structural alignment. The tridimensional alignment revealed that the two homology models did not differ in transmembrane core structure (Figure 3A), as expected from their high sequence identity; furthermore, RMSD between the two aligned GPCRs was very low (0.03 Å). We have, further, analyzed the structural similarity and capacity of discrimination of active D2-like ligands by fast docking calculations, with the Vina docking software. The structure similarity was reflected by the high correlation (R2 = 0.91, Figure 3C) of predicted binding energy of D2-like ligands docked into the homology models of hD3 and hD2L. Thus, these two homology models do not seem useful, without a structural refinement, for virtual screening directed at the recognition of selective ligands.

**Molecular Dynamics**

We have simulated for 3 ns the hD3 and hD2L homology models in a water-membrane environment that reproduces the biological milieu where these two GPCRs are located, to further discriminate their structural difference. By reporting the RMSD of protein structure from the starting homology model, both receptors differentiate in structure and reach a relative stable conformational minimum roughly after 1.25 ns (Figure 4). Total energy (Etot) and potential energy (Ep) of systems are constant during the MD simulation (Supporting Information S1) and energy values of D3 receptor are slightly lower compared to the energy of D2L subtype. We stopped simulations at 3 ns because we reached stable local minima and distinct conformations for hD3 and hD2L receptors. Longer simulations (over 30 ns) might reveal other local minima and further characterize the conformational space of these receptors; this goal, however, is beyond the aim of our study. GPCRs are in equilibrium between active and inactive conformation, and, as far as the inactive conformation is concerned, a structural marker, the “ionic lock” was described in several studies [48,49,50,51] and was also revealed in the crystal structure of eticlopride-hD3 complex (3PBL) [31]. This ionic lock involves, four conserved residues, Arg128-Asp127-Glu324-Tyr138 in hD3 (Figure 5A), and Arg132-Asp131-Glu368-Tyr142 in hD2L receptor (Figure 5B), respectively. The salt-bridges that constitute the ionic lock are retained during the 3 ns of simulation. We can assume that the conformation of receptors, that reached the relative minimum, describes the inactive state. The superimposition of the simulated hD3 and hD2L receptors confirmed the structural deviation of receptors in membrane, as the RMSD was 1.63 Å (Figure 3B). The differentiation of the two homologous receptors was further strengthened by the lower correlation (R2 = 0.74) of binding energies of D2-like ligands docked, with VINA, into hD3 and hD2L optimized structures (Figure 3D). We have measured the Cα deviation of residues belonging to the orthosteric binding pocket of receptors in order to further characterize the structural modification of hD3 and hD2L induced by the membrane environment. The deviations of these residues, comparing the initial homology models with the refined structures are reported in Table 1. The residues of binding pocket of hD2L...
receptor deviated from starting model more than residues of hD₄ subtype (Table 1). The V helix of hD₂L receptor had the greater deviation than other helices after the simulation (Supporting Information S1), involving the extracellular and intracellular side (transversal to the plane of the membrane). The VI and VII helices deviated mostly in the extracellular side and the greater deviation is shown for the VII helix (Supporting Information S1). Within the seven helices of hD₂L receptor, only IV helix had a major

Figure 1. D₂-like agonists.
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Figure 2. Re-docking eticlopride. Superimposition of eticlopride re-docked with AD4.2 (cyan lines, A) and with Vina (magenta lines, B) toward eticlopride in complex with hD₃ in the crystal structure 3PBL (green lines).
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transversal deviation and a sensible deviation along the z-axis of membrane (Supporting Information S1). Furthermore, the binding pocket of hD3 receptor was also remodeled in membrane, because there were major structural deviations involving the residues of V helix (Ser 192, Ser 193, Ser 196), VI helix (His 349) and VII helix (Tyr 375) (Tables 1 and Supporting Information S1). We further characterized the binding pocket of hD3 and hD2L, before and after refining with MD simulations, by using the web service fpocket http://fpocket.sourceforge.net/. Fpocket generates clusters of spheres to describe each pocket of a given protein; in Figure 6 we have assigned different colors to pockets of hD3 and hD2L receptors, before and after optimization. Before simulation in membrane, the binding pockets of the two receptors were very similar in shape and dimension. After simulation, the pocket of hD3 became smaller than that of hD2L and divided in three pockets (Figure 6C); the one in blue includes the orthosteric and the allosteric pockets, the one in magenta is surrounded by the extracellular loops, and the deepest and smallest pocket is colored in red. In docking calculations, we did not find poses in the red pocket, that was occupied by water molecules during MD simulation (data not shown). The pocket of hD2L after simulation became bigger than that of D3 subtype (Figure 6B and 6D). The hD2L receptor after simulation shows a big pocket (orange spheres) and a smaller pocket (magenta) located along the big one, between the III and IV helices. After simulation the red pocket of hD2L appears included within the orange one (Figure 6B and 6D). The optimized structures of hD3 and hD2L used for analysis and docking calculations were extracted randomly from one of the last frames of simulations that characterize the relative conformational equilibrium, by considering as equivalent frames belonging to the same local minimum. To confirm this assumption we randomly selected one additional frame from each local minimum of the hD3 and hD2L MD simulations. These two additional frames resulted equivalent to the previous, because, when carrying out docking of pramipexole superimposable results were obtained both in terms of binding energy (Table 2, values in brackets) and poses (data not shown). We did not carried out a clusterization of trajectories because we have reached one local minimum in each simulation. Furthermore, as reported by Yap et al [53] clusterization of GPCR trajectories, is not useful for selecting the representative structure to be used in docking calculation.

Docking

We validated the optimized structures of hD3 and hD2L receptors by docking D3-preferring receptor agonists into receptor binding pockets using AD 4.2 docking software, which provided the best result of eticlopride pose prediction in the hD3 homology model. Binding energy of agonists docked in hD3 and hD2L receptors correlates with their higher affinity for the D3 subtype (Table 2), consistent with more polar contacts of ligands docked into D3 receptor compared to ligands docked into the D2L subtype (Table 3). The experimental pKᵢ values (retrieved from http/
pdsp.med.unc.edu/free access database) of agonists were compared with the predicted values (Figure 7, see also Supporting Information S1) obtaining a good correlation as indicated by Pearson coefficients relative to hD3 and hD2L receptors equal to 0.88 and 0.83 respectively (p<0.005). Linear regression coefficients however were low (Figure 7), due to the limitations of AD4.2 in predicting absolute values of Ki, as reported by Lape et al [54] and by Yap et al [55]. Another explanation to the mentioned issue might be related to the heterogeneity in Ki determination assays. Quinpirole was not included in the regression analysis because it was an outlier, even though its predicted binding energies for hD3 and hD2L correlate with the higher affinity toward the D3 subtype. Quinpirole is a bioisoster of DPAT, among other ligands included in the regression model (Figure 1), with a tricyclic structure where the hydroxyphenyl group is substituted with a pyrazolic group. On the contrary, PD-128907, a tricyclic compound with the hydroxyphenyl group, fits in the regression model of pKi for hD3 and hD2L receptor. Another tricyclic compound included in the regression model is cis-8-OH-PBZI (PBZI), which retains the position of hydroxyl and amine groups of 7-OH-DPAT. The affinity of PBZI was determined for D2S, D3 and D4 receptors but not for D2L receptor, therefore we did not include it in the regression model for hD2L receptor. Recently, PBZI was found to not induce tolerance and slow response termination, in comparison to known agonists such as 7-OH-DPAT and pramipexole [56]. Comparing the tricyclic structures of PD-128907, PBZI and quinpirole, this latter might behave as an outlier in the chemical space, due to the substitution of the hydroxyphenyl moiety with the pyrazol condensed group.

Virtual Screening

Pramipexole is a selective D3 agonist (D2/D3 = 75.5) indicated in the treatment of early-stage Parkinson disease. This agonist was chosen as reference for building a small ligands database (89 molecules), where drug-like compounds are 70% similar to pramipexole. We carried out a virtual screening by docking these ligands into the refined hD3 and hD2L models. The top scored compound is a novel selective D2-like agonist synthesized by Ghosh et al [57] (+)-(S)-N6-Propyl-N6-(2-(4-(4-pyridin-4-yl)phenyl)ethyl)adenosine-5'-monophosphate.
nyl)piperazin-1-yl)-ethyl)-4,5,6,7-tetrahydrobenzo[d]-thiazole-2,6-
 diamine, deposited in the ZINC database with the name ZINC45254546. This compound is reported to have high affinity towards hD3 subtype (D2L/D3 = 56.5) (Table 4). ZINC45254546 (Figure 1) is an hybrid compound bearing a pramipexole moiety and a piperazin(4-phenyl(4pyridyl)) antioxidant group. This compound was re-docked with AD4.2, into hD3 and hD2L receptors. As shown in Figure 8, polar contacts involved aspartate and threonine residues in III helix and the cluster of serine residues in V helix that interact with the pramipexole group. The analysis of pose of ZINC45254546 did not show the H-bond with Asp114 in hD2L, which may explain its lower affinity toward the D2L subtype. The piperazin(4-phenyl(4pyridyl)) group interacted with part of the 2ECL in hD3 subtype and with residues of II and VII helices in hD2L receptor, that characterize the allosteric pocket. The top 30 compounds (ZINC-db code), docked into hD3 and hD2L receptors, are reported in Supporting Information S1.

Figure 6. Evolution of binding pockets of hD3 and hD2L receptor after model refinement. Pockets generated by Fpocket server are represented as colored clusters of spheres. Left panels represent hD3 (green ribbons) and right panels represent hD2L (cyan ribbons), before (A, B) and after (C, D) MD simulations. The red circles target the orthosteric binding pocket whereas the black circles highlight the allosteric binding pocket. doi:10.1371/journal.pone.0044316.g006

Table 1. Deviations of Cα of residues belonging to the orthosteric binding pocket of optimized receptors in comparison with the starting models.

| Residue   | Cα deviation (Å) | Residue   | Cα deviation (Å) |
|-----------|------------------|-----------|------------------|
| Asp 110   | 0.3              | Asp 114   | 1.3              |
| Ser 192   | 0.9              | Ser 193   | 1.3              |
| Ser 193   | 0.9              | Ser 194   | 1.0              |
| Ser 196   | 1.3              | Ser 197   | 3.2              |
| Trp 342   | 0.3              | Trp 386   | 1.5              |
| Phe 345   | 0.3              | Phe 389   | 1.8              |
| Phe 346   | 0.3              | Phe 390   | 0.9              |
| His 349   | 0.6              | His 393   | 1.8              |
| Tyr 375   | 1.2              | Tyr 416   | 0.9              |

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Table 2. Predicted binding energy (Autodock 4.2) of D3 agonists towards hD3 and hD2 receptors. Experimental Kᵢ (exp. Kᵢ) with respective references are also shown.

| D3 agonist [reference] | hD3 E(binding) (kcal/mol) | hD2 E(binding) (kcal/mol) | hD3 exp. Kᵢ (nM) | hD2 exp. Kᵢ (nM) |
|------------------------|---------------------------|---------------------------|------------------|------------------|
| Dopamine               | −6.5                      | −6.0                      | 32.5(1)          | 598(1)           |
| r-7-OH-DPAT [61]       | −7.7                      | −6.4                      | 1.58             | 158              |
| r-7-OH-PIPAT [19]      | −8.4                      | −7.3                      | 2.9(2)           | 142(2)           |
| Pramipexole [62]       | −7.1                      | −6.6                      | 10.5             | 790              |
| Pramipexole(3)         | (−7.1)                    | (−6.4)                    |                  |                  |
| Ropinirole [62]        | −7.0                      | −6.4                      | 37.2             | 933              |
| Rotigotine [63]        | −8.4                      | −7.4                      | 0.71             | 13.5             |
| Quinpirole [64]        | −7.6                      | −6.6                      | 39               | 1402             |
| PD 128907 [65]         | −7.7                      | −6.0                      | 3.1              | 1573             |
| cis-8-OH-PBZI [66]     | −7.1                      | ND                        | 27.4             | ND               |

(1)Average value from PDSP database: http://pdsp.med.unc.edu/indexR.html.
(2)The Kᵢ is reported for the racemic 7-OH-PIPAT.
(3)Pramipexole re-docked in two other frames of hD3 and hD2L receptor; see also text.
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Figure 7. Correlation of predicted pKᵢ and experimental pKᵢ values. Plots of D₃ preferring agonists docked toward hD₃ (A) and hD₂L (B) receptors: a. dopamine; b. 7-OH-DPAT; c. 7-OH-PIPAT; d. pramipexole; e. quinpirole; f. ropinirole; g. rotigotine; h. PD 128,907; i. cis-8-OH-PBZI; j. ZINC45254546.
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Table 3. Ligand protein-interaction of D₃-preferring receptor agonists docked with AD4.2.

| Ligands          | hD₃ Hydrogen bonds-polar contacts | hD₃ Hydrophobic contacts | hD₃L Hydrogen bonds-polar contacts | hD₃L Hydrophobic contacts |
|------------------|----------------------------------|-------------------------|-----------------------------------|---------------------------|
| Dopamine         | Asp 110, Thr 115, Ser 192, Ser 196 | Ile 183, Phe 345, His 349 | Asp 114, Ser 194                  | Val 115, His 393, Phe 389, Phe 390 |
| r-7-OH-DPAT      | Asp 110, Ser 192, Ser 196, Thr 115 | Ile 183, Phe 345, His 349 | Asp 114, Ser 193                  | Val 111, Phe 110, Ile 184, Phe 390 |
| r-7-OH-PIPAT     | Asp 110, Val 111 (C = O of peptide bond), Thr 115, Ser 192. | Val 111, Val 107, Ile 183, Trp 342, Phe 345, His 349 | Asp 114, Val 190 (C = O of peptide bond), Ser 193. | Val 111, Phe 110, Ile 184, Phe 390. |
| Pramipexole      | Asp 110, Thr 115, Ser 192, Ser 196 | Val 111, Trp 342, Phe 345, Thr 369. | Asp 114, Val 190 (C = O of peptide bond), Ser 194. | Phe 110, Val 111, Phe 390, His 393. |
| Ropinirole       | Asp 110, Ser 192                   | Val 189, Trp 342, Phe 345, His 349 | Asp 114, Ser 193                  | Val 111, Phe 110, Val 115, Phe 390, His 393 |
| Rotigotine       | Asp 110, Ser 192                   | Val 107, Phe 106, Phe 345, Phe 346, His 349 | Asp 114 | Phe 110, Val 111, Val 115, Ile 184, Phe 390, His 393 |
| Quinpirole       | Asp 110, Ser 192                   | Val 111, Ile 183, Trp 342, Phe 345, Thr 369, Tyr 373. | Asp 114 | Val 115, Trp 386, Phe 389, Gly 415, Tyr 416. |
| PD128907         | Asp 110, Ser 192                   | Val 111, Ile 183, Phe 188, Trp 342, Phe 345, Phe 346, Thr 369, Tyr 373. | Asp 114 | Val 111, Phe 389, His 393. |
| cis-8-OH-PBZI    | Asp 110, Ser 192, Ser 196, Thr 115 | Val 111, Ile 183, Trp 342, Phe 346, Thr 373, Thr 369. | *ND | *ND |

*ND = Not Determined.
Residues involved in H-Bonds are underlined.
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Discussion

In the present study we have successfully modeled and optimized the structure of two high homologous GPCRs, the hD₃ and hD₂L receptors. The homology modeling is a powerful tool in the prediction of protein structure. The strength of this methodology is related to the sequence identity shared between the target and the template protein: the highest sequence identity determines the best structure model. We built and validated the homology models of hD₃ and hD₂L receptor using the x-ray structure of hD₃ receptor, a lysozyme-chimera protein. The high sequence identity shared by these two receptors did not allow us to differentiate their homology models that were therefore unsuitable for prediction of binding energies and subtype selectivity of D₂-like ligands. The high structure similarity of hD₃ and hD₂L arises from the energy minimization process, and represents a weakness in the homology modeling approach. Usually, in homology modeling, the energy optimization of the modeled protein structure is performed by energy minimization in vacuo, with some exceptions such as the GPCRRD server http://zhanglab.ccmb.umich.edu/
GPCRDRD/ GPCRDRD carries out a pipeline of structural optimizations of homology models, with a final MD simulation: Fragment-Guided Molecular Dynamics (FD-MD), which takes into account knowledge-based (H-bonds and positional restraints) and physics-based atomic potentials (AMBER99 forcefield) [58,59]. So far protein-lipid and protein-water explicit interactions, based on empirical physics-based atomic potentials, are not taken into account by homology modeling software. Thus, we attempted to optimize the structure of the hD3 and hD2L models by MD in an explicit water-membrane environment, reaching a local conformational minimum within 3 ns. The MD simulations led to structural adaptation and differentiation of the two receptors in membrane, enabling the prediction of trends of pKᵢ values and the modeling of ligand-protein interactions of D₃-prefering receptor agonists. Moreover, the refined models were useful in the identification, by a virtual screening approach, of an agonist (ZINC45254546) referred to be selective for D₃ over D₂ [57]. Our results are consistent with the findings of Chien et al [26]; the hD₃ homology model we built was validated by docking eticlopride and by obtaining with AD 4.2 a pose highly similar to the one in the x-ray structure 3PBL. Because the ionic lock, a marker of inactive state described in 3PBL, was retained during MD simulations in both hD₃ and hD₂L receptors, we can assume that refined models represent an inactive state of the receptor. Moreover, we modeled both disulfide bridges solved in 3PBL in hD₃ model and we modeled just one disulfide bridge, the canonical one, in hD₂L. We made this choice because the conserved cysteine residues in the 3ECL, Cys 399 and Cys 401, are separated just by one residue Asp 400, leading to a high constrained loop in the case a disulfide bridge is formed. The lack of the accessory disulfide bridge in the 3ECL might have influenced the dynamics of hD₂L receptor, leading to the swelling of its binding pocket, in comparison to the hD₃ which is restrained by two disulfide bridges. Wang et al [60] have predicted the structural differences of hD₃ and hD₂ receptors. The homology models of these GPCRs were built in complex with haloperidol (previously aligned to the β₂-adrenergic inverse agonist s-carazolol), using the crystal structure of β₂-adrenergic receptor [2RH1]; the complexes were subsequently simulated in a POPC bilayer for 1.5 ns. Haloperidol in complex with simulated D₂ and D₃ receptors was also used to carry out 3D-QSAR studies using 163 compounds. These authors [35] concluded that the higher affinity of bigger ligands for D₂ receptor over D₃ subtype is related to the shape of binding pocket, which is shallower in D₂ receptor. We found that the binding pocket of hD₃ receptor, after adapting in the membrane environment, significantly deviates from the initial homology model, becoming smaller and partitioned. The binding pocket of hD₃ in membrane environment is also smaller than the one of hD₂L receptor. We carried out docking calculations rather than 3D-QSAR (ligand-based method) because we considered our refined models highly predictive due to the crystal structure of hD₃ receptor, used as template for homology modeling. Docking calculations (structure-based method) are strictly related to the reliability of the receptor structure, and we obtained a good correlation of experimental and computed Kᵢ values for agonists docked into hD₃ and hD₂L binding sites. Although the prediction of absolute Kᵢ values is a difficult task, AD 4.2 was a powerful tool in order to validate homology model of hD₃ receptor (eticlopride re-docking) as well as to validate the refined models by MD simulations. In fact, the predicted trend of Kᵢ values is well correlated (high Pearson coefficients) with the experimental trend. This correlation was carried out with aminotetrailine derivatives, a congeneric chemical class that does not include quinpirole. This latter is a preferential D₃ agonist, but behaved as an outlier in the chemical space of docked ligands, due to the tricyclic structure and the pyrazole moiety. Nevertheless, our optimized models were able to predict the affinity of quinpirole higher for D₃ than for D₂ receptor. In conclusion, the computational approach, totally structure-based, adopted in the present study is able to build and refine structure models of homologous dopamine receptors that may be of interest for structure-based drug discovery of selective dopaminergic ligands, potentially useful to treat neurological, psychiatric and ocular disorders.

Supporting Information

Supporting Information S1 Figure S1: Energy plots of systems. Potential energy (E_pot) and total energy (E_tot) of hD₂L and hD₃ receptors. Table S1: Cz deviations of transmembrane helices (TM) of D₂ and D₃ simulated receptors from the starting models. Cz deviation values were determined by structural alignment of each helix of the model and of the optimized structure. Figure S2: Deviation of helices of optimized hD₃ receptor (cyan cartoon) respect the starting model (yellow cartoon). The upper side of the figure corresponds to the extracellular side. Table S2: Computed pKᵢ for ligands docked into hD₃ and hD₂L receptors. Values are reported for ligands inserted in the regressions represented in Figure 7. Figure S3: Superimposition of template (3PBL)-homology model- optimized model of hD₃ receptor and hD₂L receptor. The template structure (green cartoon) is the A chain of hD₃ receptor crystal structure (3BPL). The cyan cartoon corresponds to the homology model of hD₃ receptor, the yellow cartoon corresponds to the homology model of hD₂L receptor. The optimized models of hD₃ and hD₂L receptor are respectively the magenta and orange cartoons. (DOCX)

Supporting Information S2 Supplemental files (.pdb files) contained in the compressed directory File S2 include poses of

Table 4. Virtual Screening. Top scored compound ZINC45254546.

|      | hD₃          | hD₂L         |
|------|--------------|--------------|
| Vina (Kcal/mol) | −8.7         | −8.1         |
| AD4.2 (Kcal/mol) | −8.8         | −7.98        |
| Exp. Kᵢ (nM)   | 4.78         | 270          |
| H-bonds and Polar contacts | Asp 110, Thr 115, Ser 196, Ser 182, Ser 197, Ser 193, Thr 119, Thr 193, Thr 119 |
| Hydrophobic interactions | Val 111, Ile 183, Phe 345, Phe 346, His 349, Tyr 365, Pro 362, Thr 369, Leu 94, Val 91, Val 111, Ile 184, Val 115, Phe 198, Phe 389, Phe 390, His 393, Thr 412, Tyr 416 |

Residues involved in H-bonds are underlined.
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ligands, shown in Figure 1, docked into hD3 and hD2L, optimized receptors, whose.pdb files are also included in File S2. All.pdb files can be visualized with Open Pymol. Files named ligand_D2L.pdb correspond to poses of ligand docked into hD2L receptor, whereas files named ligand_D3.pdb correspond to poses into hD3 receptor. The optimized structure of hD3 and hD2L receptor are named respectively opt_D3_receptor.pdb and opt_D2L_receptor.

Author Contributions
Conceived and designed the experiments: CBMP CB GML. Performed the experiments: CBMP CB. Analyzed the data: CBMP CB. Contributed reagents/materials/analysis tools: CBMP CB. Wrote the paper: CBMP CB. }

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