Active-State Models of Ternary GPCR Complexes: Determinants of Selective Receptor-G-Protein Coupling

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Abstract

Based on the recently described crystal structure of the β2 adrenergic receptor - Gs-protein complex, we report the first molecular-dynamics simulations of ternary GPCR complexes designed to identify the selectivity determinants for receptor-G-protein binding. Long-term molecular dynamics simulations of agonist-bound β2AR-Gs and D2R-Gs complexes embedded in a hydrated bilayer environment and computational alanine-scanning mutagenesis identified distinct residues of the N-terminal region of intracellular loop 3 to be crucial for coupling selectivity. Within the G-protein, specific amino acids of the α5 helix, the C-terminus of the Gs-subunit and the regions around αNβ1 and α4β6 were found to determine receptor recognition. Knowledge of these determinants of receptor-protein binding selectivity is essential for designing drugs that target specific receptor/G-protein combinations.

Introduction

G-protein-coupled receptors (GPCRs) are proteins that enable signal transduction through biological membranes. The more than 800 GPCRs (including receptors for olfaction and taste) constitute the largest family of membrane proteins in the human genome [1]. GPCRs show pronounced structural variety in their binding pocket and can thus be activated by diverse extracellular signals including photon-induced changes in ligand conformation, small molecules, peptides and proteins [2]. Agonist binding causes structural rearrangements in the intracellular part of the receptor [3–9] that enable binding of a heterotrimeric G-protein and thus formation of the ternary complex consisting of agonist, receptor and G-protein [10]. The ternary complex induces the transmission of signals that activate both distinct physiological processes involving sensory impressions such as vision, smell and taste and neurological, cardiovascular, endocrine and reproductive functions that make GPCRs (and G-proteins) important targets for drug design [11].

After the structural characterization of the β2-adrenergic receptor (β2AR) bound to an antagonist [12,13] and the first agonist-β2AR complexes [14,15], the crystal structure of the β2AR together with its signal-transducing Gs-protein was determined by Brian Kobilka and his team [16]. This spectacular work indicated a receptor conformation that was similar to the antagonist-bound form [14]. Only in the presence of a G-protein simulating nanobody [13] or the G-protein itself [16], could the rigid body movements described above be observed. Recently, NMR experiments investigating the dynamic behavior of β2AR emphasized the fundamental role of an intracellular binding pocket in the stabilization of a fully-activated receptor conformation [17].

The crystal structure provides a physiological, atomistic template of a fully-activated G-protein-coupled receptor bound to and stabilizing a nucleotide-free G-protein. It represents a valuable template for homology modeling studies that explore high-affinity active-state binding sites of GPCR-G-protein complexes. Active-state homology models can be of great importance for identifying new agonist lead-structures, for example in docking campaigns [18]. Because many GPCRs can bind multiple G-protein-subtypes, models of individual receptor-G-protein complexes are needed to design functionally selective drugs inducing the activation of a particular G-protein to a higher extend than coupling to alternative G-protein subtypes.

Herein, we describe the first active-state homology model of a G-protein-coupled receptor in complex with its preferred G-protein based on the crystal structure of the β2-adrenergic receptor in complex with the Gs-protein [16]. In order to identify the amino acids responsible for coupling selectivity between GPCRs and G-proteins, we examined the protein-protein interface of two different ternary complexes, the agonist-bound β2AR-Gs, crystal structure and, based on the β2AR-Gs structure, two homology models of the dopaminergic D2 receptor (D2R), a drug target of particular interest for the treatment of neuropsychiatric disorders including Parkinson’s disease and schizophrenia [19], in complex

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with dopamine and G_{\alpha_i}. We carried out one \(\mu s\) molecular-dynamics simulations in a hydrated bilayer built of dioleoylphosphatidylcholine-lipids (DOPC) for each, and investigated the receptor G-protein interface by computational alanine scanning mutagenesis.

**Results and Discussion**

**Active-state Homology Models of D2R-G_{\alpha_i}**

According to Kobylka et al. [16], the "active state of a GPCR can be defined as that conformation that couples to and stabilizes a nucleotide-free G-protein." We therefore used the crystal structure of the \(\beta2AR-G_{\alpha_i}\)-complex (PDB-ID: 3SN6) as a starting point for active-state homology models of D2R in complex with the nucleotide-free state of G_{\alpha_i}. We created alignments for the separated receptors and the G-proteins, combined them and subsequently started the modeling process using MODELLER 9v4. A more detailed description of the modeling process is provided in the Methods section. The models exhibited two different rotamer conformations of residue His3936.55 in D2R with the side chain of histidine pointing either to the extracellular or to the intracellular part of the receptor (Figure 1). His3936.55 has been shown to play a significant role in ligand binding and signaling bias at dopamine receptors [20–22] and that, in principal, both conformations are possible [23]. Therefore in the following studies, we decided to select two models of the D2R-G_{\alpha_i}-complex with both rotamer conformations of His3936.55, which are referred to in the following as D2\(^{\alpha_i}\)-R-G_{\alpha_i}, and D2\(^{\beta_{\alpha_i}}\)-R-G_{\alpha_i}. The physiological agonist dopamine was docked manually into D2\(^{\alpha_i}\)-R-G_{\alpha_i} and D2\(^{\beta_{\alpha_i}}\)-R-G_{\alpha_i} in a way that the positively charged ammonium head group forms a salt bridge to Asp1143.32 and that hydrogen bonds between the catechol moiety of dopamine and the side chains of Ser1935.42 and Ser1975.46 of D2R become feasible (Figure 1). These serine residues, Ser1935.42 and Ser1975.46, together with Ser1945.44, have been shown to be crucial for high-affinity catecholamine binding and for an effective receptor-G-protein coupling [24,25].

A agonist binding of GPCRs leads to major structural changes within the receptors and the G-proteins that are consistent with the conformation of our active-state D2R-G_{\alpha_i}-complexes (Figure S1).

**Molecular-dynamics Simulations**

Three ternary complexes, \(\beta2AR-BI167107-G_{\alpha_i}\) and D2\(^{\alpha_i}\)-R/ D2\(^{\beta_{\alpha_i}}\)-R-dopamine-G_{\alpha_i}, were successfully embedded into a hydrated DOPC-bilayer. We cleared a space for the initial insertion of the protein structures into the bilayer by removing DOPC-molecules from the bulk of the membrane (Figure S2a). A careful equilibration procedure was used to close the resulting gap between GPCRs and DOPC-residues (Figure S2b, c) without water molecules flooding this gap. The resulting complexes were subsequently submitted to molecular-dynamics (MD) simulations for one \(\mu s\) each, with the interior of the DOPC-bilayer remaining free of water throughout the simulations (Figure S3). The long simulation time of one \(\mu s\) for each complex was chosen to ensure the formation of sufficiently stable amino-acid contacts between the proteins in order to be able to elucidate amino acids that appear in the interface of GPCRs and G-proteins reliably.

All complexes remained very stable throughout the MD simulations showing low RMSD values for every member of the ternary complexes (Figure S4). As the G-proteins were not stabilized by membrane lipids, they showed higher atomic fluctuations than the receptor moieties (Figure S5). Substantial mobility was observed for the helical subunits of G_{\alpha_i} and G_{\alpha_i}, G_{\alpha_i}AH and G_{\alpha_i}AH, which have previously been shown to become highly flexible in their nucleotide-free state [26,27]. Comparing the atomic fluctuations of the two D2R-G_{\alpha_i}-complexes, we observed higher values for the D2\(^{\alpha_i}\)-R-G_{\alpha_i}-simulation (Figure S5), which were connected to a whole-body movement of G_{\alpha_i} starting at the lower part of the \(x_{5}\)-helix, but leaving the majority of \(x_{5}\) and its C-terminus unaffected (Figure S6a, b). The movement of G_{\alpha_i} originates in the enhanced flexibility of open ends in the N-terminal IL3, which is mainly associated with the absence of the bulk of IL3 (Figure S5). This enhanced flexibility causes a loss of ionic interactions between residues from the N-terminal part of IL3 and residues from the area around \(\alpha_4-\beta_6\), which finally results in a displacement of G_{\alpha_i} around helix \(\alpha_4\) in the D2\(^{\alpha_i}\)-R-G_{\alpha_i}-simulation compared to the D2\(^{\beta_{\alpha_i}}\)-R-G_{\alpha_i}-complex. As this conformation appeared to be stable for the remainder of the simulation and did not lead to the separation of D2R and G_{\alpha_i} (Figure S4, S7), we continued investigating both D2R-G_{\alpha_i}-complexes. Additionally, our data give no indication for any displacements of GPCRs and G-proteins other than the one described for the D2\(^{\alpha_i}\)-R-G_{\alpha_i}-complex.

The agonists BI167107 and dopamine in the \(\beta2AR-G_{\alpha_i}\)-complex and in the D2R-G_{\alpha_i}-complexes, respectively, are largely enclosed in their binding pockets. In the \(\beta2AR-G_{\alpha_i}\)-complex, BI167107 maintained its interactions with residues TM2, TM3, TM5, TM6 and TM7, most of which were already present in the crystal structure (Figure 2a, b). In the case of the D2R-G_{\alpha_i}-complexes, dopamine showed a different orientation of its catechol moiety within the binding pockets. Whereas only the meta-hydroxy group of dopamine formed a hydrogen bond to Ser1935.42 in the D2\(^{\beta_{\alpha_i}}\)-R-G_{\alpha_i}-complex, both, the meta- and para-hydroxy groups of dopamine were involved in the formation of hydrogen bonds to Ser1935.42 and Ser1975.46 of D2\(^{\beta_{\alpha_i}}\)-R, respectively (Figure 2c, d).

This behavior may be associated with changes in the rotamer conformation of residue His3936.55 throughout the D2R-G_{\alpha_i}-simulations, where its side chain adopts three distinct dihedral angles, referred to as states 1, 2 and 3 (Figure 3). In state 1, the side chain of His3936.55 points towards the intracellular site of the receptor into the direction of TM7 (the initial conformation of the D2\(^{\beta_{\alpha_i}}\)-R-G_{\alpha_i}-complex), where it is stabilized by an interaction to

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**Figure 1. Initial conformation of dopamine in the D2R-G_{\alpha_i}-complexes.** The backbone of D2R is shown as green ribbon, with important amino acids (indicated as green sticks) that stabilize the ligand dopamine in its initial conformation. Dopamine is represented as orange sticks and stabilized by ionic interactions to D1143.32 and hydrogen bonds to S1935.42 and S1975.46. The second conformation of residue H3936.55 is shown as red sticks. doi:10.1371/journal.pone.0067244.g001
residue Tyr4087.35 of upper TM7. State 2 shows the side chain of histidine pointing towards the extracellular part of the receptor (the initial conformation of the D2<sub>Down</sub>/UpR-complex and the one observed in the crystal structure of D3R), where it regains spatial proximity to Tyr4087.35 of TM7. The side chain is again oriented towards the intracellular site of the receptor in state 3, but now points in the direction of TM5, which enables a hydrogen bond to be formed to residue Ser1935.43. We assume that the dihedral angle of His3936.55 causes structural differences within the binding pocket of D2R, which lead to different conformations with respect to ligand binding. Structural connections between His6.55, Tyr7.35, TM5-serines and ligands that are able to discriminate between different downstream signaling pathways have been shown to be involved in biased signaling [23]. The agonist dopamine, which cannot cause functional selectivity, does not prevent the side chain of His3936.55 from cycling between its possible rotamer conformations. Sterically more demanding ligands may lock His3936.55 in one distinct rotamer conformation and thus trigger the activation of one distinct pathway. Therefore, further MD-simulations with selected ligands are necessary to elucidate the impact of His3936.55 on functionally selective signaling.

The Receptor-G-protein Interface

Our μ MD-simulations were carried out in order to identify stable amino-acid contact sites between the receptors and their G-proteins that are maintained for long periods. Early experimental work, which focused on elucidating the interface between rhodopsin and its G-protein transducin using synthetic peptides that correspond to different regions of rhodopsin and transducin, identified the intracellular loops 2 and 3, the junction between TM7 and helix 8 of rhodopsin [28] and the area around α<sub>4</sub>b<sub>6</sub> and the C-terminal helix of transducin’s Gα subunit, Gα<sub>t</sub> [29], as important contact sites between the two binding partners. These contact areas were further strengthened by a disulfide cross-linking study using the muscarinic M3 receptor and Gα<sub>s</sub> [30]. A first structural glimpse of the amino acids involved in binding GPCRs to G-proteins was provided by crystallizing light-activated opsin together with a synthetic peptide (Gα<sub>CT</sub>, residues ILENLKDCGLF) derived from the C-terminus of Gα<sub>t</sub> [31]. By mutating the residues in Gα<sub>CT</sub> into the corresponding amino acids of Gα<sub>s</sub>, we were able to delineate its interactions with β2AR [9]. Now, with the crystal structure of an entire ternary β2AR-Gα<sub>s</sub> complex at hand, we have an excellent framework for investigating active-state models of structurally unknown ternary GPCR-complexes via computational methods.
The trajectories of the MD simulations were therefore screened for amino-acid contacts between the receptors and the appropriate G-proteins. The receptor-G-protein interfaces are shown in Figure 4 as individual alignments for the receptors and for the G-proteins. Amino acids are highlighted in the alignment when at least one atom of an amino acid approaches at least one atom of another amino acid closer than 3.5 Å and when this interaction is found in more than 50% of the simulation. Detailed connection tables are provided in the (Table S1, S2, S3).

The receptor-G-protein interface of these fully-activated, nucleotide-free ternary complexes is comprised of homologous regions within the β2AR-Gαs-complex and the D2R-Gαi-complexes. The amino-acid contacts within the two D2R-Gαi-simulations were found to be highly congruent, despite the differences concerning the displacement of Gαi discussed above (Figure S6). GPCR contacts include the area around IL2, the N- and C-terminal parts of IL3 and the junction of TM7 and helix 8. The latter area only emerged as a contact region during the MD simulations and is not visible in the crystal structure of the β2AR-Gαs-complex. This observation underlines the importance of dynamic techniques such as MD simulation, which are not limited to a static snapshot of the protein. The G-protein contact regions consist of the αNβ1-loop, the area around β2-β3, the area around αβ3-β6 (with different distributions of the contact residues for the β2AR-Gαs- and the D2R-Gαi-complexes) and the C-terminal αβ5-helix together with its C-terminus.

Additional information about the receptor-G-protein interfaces is given by highlighting the individual residues that appear in these interfaces with different colors that show the number of individual contacts from one residue to others: the darker the color (from yellow over green to blue) the more neighbors an amino acid has and the more important it is likely to be for receptor-G-protein coupling. Thus, the C-terminal domain of Gαi, where high densities of tightly packed amino acids occur, can be assigned an outstanding role for complex stabilization and coupling selectivity arising from the G-protein. This is because the C-terminal αβ5-helix together with its extreme C-terminus is incorporated in the cavity formed by the outward movement of TM6 during receptor activation, which enables pronounced interactions with all of the contact regions of the GPCRs depicted. On the side of the receptors, we observed pronounced interactions for residues belonging to the areas around IL2 and the junction of the distal part of TM5 connected to the N-terminal part of IL3.

Computational Alanine-scanning Mutagenesis

To elucidate the importance of each amino acid that appears in the interface between receptors and G-proteins, we carried out computational alanine-scanning mutagenesis of the β2AR-Gαs- and the D2R-Gαi-interfaces. This approach has been shown to be a valuable tool for estimating the contribution of individual amino acids to the stabilization of protein-protein interactions [32] and to be able to reproduce experimental investigations qualitatively [33]. We therefore used the MMGBSA-method (Molecular Mechanics-Generalized Born Surface Area) [34], implemented in MM/PBSA/FEP [35], to calculate the relative binding free energy changes (ΔΔG) between alanine-mutant complexes and the corresponding wild-type complexes in order to identify so-called hot-spot residues within the GPCR-G-protein interfaces that contribute to both coupling affinity and selectivity.
In a first step, we omitted water and membrane molecules and calculated the binding free energies ($\Delta G$) of the $\beta_2$AR-G$\alpha_s$- and the D2 UpR/D2DownR-G$\alpha_i$-interfaces using the GBSA-method within MMPBSA.py in order to prove that the complex is energetically favorable and that the energy values remain generally consistent over the time scales investigated. Conformationally stable time periods within the three ternary complex trajectories were identified based on RMS deviations (Figure S4) and used to generate the required trajectories for the receptor- and the G-protein-parts with intervals of 500 ps between snapshots. Our calculations showed consistently negative $\Delta G$-values for the systems on the time scales investigated, which indicates energetically favorable interactions between receptors and G-proteins (Figure S8). We subsequently performed computational alanine-scanning mutagenesis for the amino acid residues within the receptor-G-protein interfaces of $\beta_2$AR-G$\alpha_s$- and the D2 UpR/D2DownR-G$\alpha_i$-complexes that are highlighted in Figure 4, except for alanine-, glycine- and the C-terminal residues L380 and F354 from G$\alpha_s$ and G$\alpha_i$, respectively. In cases where only one amino acid of the D2 UpR/D2DownR-G$\alpha_i$-complexes constitutes a contact residue, we nevertheless performed alanine scanning on both amino acids. Important results of the alanine scan are shown in Figure 5, the complete results are provided in the (Table S4). In general, a positive value for the binding free energy change ($\Delta\Delta G$) is associated with an amino acid that contributes to stabilizing the ternary complex, and vice versa.

For the $\beta_2$AR-G$\alpha_i$-system, we found that residues R131, I135, F139, Q229, K232, I233, E237, K270 and R333 from $\beta_2$AR and H41, Y344, D367, I369, Q370, R371, H373, L374, R375, Y477, E378 and L379 from G$\alpha_i$ stabilize the receptor-G-protein interface. Among these residues, F139 from IL2, Q229 and E237 from TM5-IL3 and R333 from helix 8 have been found to...

Figure 4. Alignment of the amino-acid contacts between receptors and G-proteins. Individual alignments for the receptors and the G-proteins are shown. A colored background indicates that the residue forms contacts to other amino acids (yellow: 1 or 2 contacts; green: 3 or 4 contacts; blue: at least 5 contacts). Red letters indicate residues involved in ionic interactions, whereas dotted underlines indicate contacts present in the crystal structure of $\beta_2$AR-G$\alpha_s$. doi:10.1371/journal.pone.0067244.g004
Figure 5. Summary of selectivity determining amino acids within the β2AR-Gαs- and the D2R-Gαi-complexes and representative values of the alanine scanning mutagenesis. The grey columns in the middle refer to the regions within GPCRs and G-proteins, to which the mentioned amino acids belong. Amino acids in italic letters have not been mutated in the computational alanine scanning (n.d.). Blue, green and red bars show the binding free energy differences of the alanine scanning mutagenesis for the β2AR-Gαs complex and the D2DownR-Gαi and the D2UpR-Gαi-complexes, respectively. The orange and red rectangles besides the amino acids correspond to hydrophobic or polar interactions to other residues, respectively.

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be of major importance for β2AR, whereas D367 and R375 from the C-terminus of the Gαs-subunit are important for Gαs. F139 interacts tightly with a hydrophobic pocket comprised of residues H41, V203, F205, F362, C365, R366 and I369 from Gαs (Figure 6f, Table S1) and thus stabilizes the interface of IL2, αN-β1, β2-β3 and α5. It has been shown that mutating F139 to alanine in β2AR prevents activation of adenyl cyclase by Gαs and that, in general, a bulky amino acid is necessary in this position for effective receptor-G-protein coupling [36]. Residue Q229 from the N-terminal IL3 forms the center of an extended hydrogen-bond network to residues D367, Q370 and R371 of the α5-helix of Gαs and K232 from TM5 of β2AR (Figure 6e, Table S1). Residue E237 from IL3 and R333 from H8 of β2AR are involved in salt bridges to residues R375 from the α5-helix and E378 from the C-terminus of Gαs, respectively (Figure 6c, Table S1).

For the two D2R-Gαs-simulation systems, amino acids important for receptor-G-protein-binding were found to be, in general, qualitatively comparable between D2Down-R-Gαs and D2Up-R-Gαs. The main difference is caused by the movement of Gαs within the D2\textsuperscript{1L/1R}-Gαs-complex discussed above, which weakens the interactions between residues from the extreme N-terminal IL3 and the area around α4-β6. Taken together, residues R132, V136, M140, Y142, R145, R150, R219, R222, K226\textsubscript{Down}, R227\textsubscript{Down}, K232, K234, R247, K345, R371 of the Gαs-subunit are involved in salt bridges to residues R375 from the α5-helix and E378 from the C-terminus of Gαs, respectively (Figure 6c, Table S1).

The most striking difference between the D2 DownR-Gαs and D2Up-R-Gαs-complexes lies in the conformation of residue R132 from TM3 (Figure 6a, d). Whereas the side chain of R132 points “downwards” in the direction of the C-terminal α5-helix of Gαs in the D2\textsuperscript{1L/1R}-Gαs-complex, its side chain reaches out directly towards the junction of TM7/H8 in the D2\textsuperscript{1L/1R}-Gαs-complex. R132 forms a salt bridge to residue D350 from the C-terminus of Gαs in D2\textsuperscript{1L/1R}-Gαs. In contrast, R132 and D350 do not show direct D2\textsuperscript{1L/1R}-Gαs-interactions. Thus, the conformation of R132 is stabilized by residue F429 from H6 of D2R and D350 of Gαs forms a hydrogen bond to residue N430 of D2R.

### Selectivity Determinants

Selectivity of a GPCR for a distinct G-protein (or vice versa) arises from structural differences at the interacting epitopes. Figure 5 provides a direct comparison between residues of the β2AR-Gαs- and the D2R-Gαs-complexes that participate in stabilizing the receptor-G-protein interfaces while showing sequence differences at the same time. Highlighted amino acids of β2AR and D2R are suggested to be crucial for coupling to Gαs and Gαs-proteins, respectively, as they exhibit a high degree of sequence conservation within the subfamily of amineergic Gαs and Gαs coupled receptors, which is depicted in Figure 7.

Significant amino acids that control selective receptor-G-protein coupling are located mainly at the intracellular end of TM5 and the N-terminal region of IL3, which comprise a coupling domain for the C- and N-terminal part of Gαs and the α4/β6 domain (Figure 5). Interactions of β2AR with the C-terminus of Gαs are supported by residues from TM3/IL2, TM6 and TM7/H8. Among these residues, I135, A226, Q229, I233, E237, T274 and I278 represent a strongly conserved feature of amineergic GPCRs that couple preferentially to Gαs (Figure 7). The equivalent of A226 in TM5 is represented by an alanine residue for every Gαs-coupling amine receptor, but differs within the Gαs-coupled subfamily. The C-terminal parts of Gαs differ significantly (Figure 4, 6). Residues V377, E378 and L380 as well as D350, C351, G352 and F354 in Gαs and Gαs, respectively, are differently stabilized within their GPCR-pockets and lead to a different orientation of their C-termini (Figure 8). Together with residues from the lower parts of the α5-helix (Q370, R371, H373 and R375 in Gαs and E344 and N347 in Gαs), which interact with amino acids from the N-terminal part of IL3 of the receptors, they constitute, in general, the main determinant of coupling selectivity of G-proteins. The importance of these regions is supported by mutational studies [37–40]. In agreement with functional experiments with artificial model proteins indicating the importance of the N-terminal part of IL3 for D2R coupling [41,42], the selectivity-determining areas of the D2R-Gαs-complexes were found to be located in the intracellular TM5/terminal IL3-region of D2R and the C-terminal part of Gαs. Selective coupling is supported by the junction of TM3 and IL2, the C-terminal TM6 and the junction of TM7 and helix 8 (Figure 5) when the major amino acids of GPCRs that couple mainly to Gαs were shown to be a valine residue (V136 in D2R) in TM3 and two residues from TM7/H8, F429 and N430 in D2R (Figure 7).

The most striking difference between the β2AR-Gαs-complex and the D2R-Gαs-complexes was identified for the interaction of the GPCRs’ intracellular loop 2 and the domains αN/β1 and α4/β6 of the G-proteins. Thus, the intracellular loop 2 of β2AR presents a phenylalanine (F139) interacting with a hydrophobic pocket formed by residues from αN/β1, β2-β3 and α5 of Gαs (Figure 6f). Especially the aromatic amino acid H41 and F205 from Gαs are suggested to enable a highly efficient stabilization of the aromatic residue F139 or, to a lesser extent, other bulky, hydrophobic residues in the equivalent position of IL2 in other GPCRs (Figure 5, 7). In contrast to the hydrophobic interaction of β2AR and Gαs, D2R and Gαs form ionic interactions between basic amino acids of D2R and negatively charged amino acids of Gαs. Ionic contacts involve arginine residues of IL2/TM5 (R145, R150) and TM5/IL3 (R219, K226, R227) of D2R and glutamate residues of αN/β1 (E25, E26) and α4/β6 (E308, E318) of Gαs. The
importance of these basic amino acids in D2R is proposed to be a general determinant of coupling selectivity towards Gi, as the structures of Gi preferring aminergic GPCRs exhibit homologous residues in the corresponding positions (Figure 7).

Conclusions

To evaluate receptor binding and activation of unexplored GPCR subtypes and to understand the variety of functionally relevant conformations better, the recent crystal structure of the ternary b2AR-Gas-complex must be complemented by dynamic techniques such as molecular-dynamics simulations, NMR or mass spectroscopy. Because many GPCRs are able to bind more than one G-protein-subtype, models of individual receptor-G-protein complexes will facilitate the rational design of functionally selective drugs inducing the activation of a particular G-protein to a higher extend than coupling to alternative G-protein subtypes. Activation of multiple G-protein dependent and independent pathways and the existence of functionally biased ligands have been demonstrated for the pharmacologically relevant D2R [43–45]. The different coupling characteristics of the Gas-subunits Gai and Gao towards D2R are associated with subtle sequence differences within their GPCR binding interfaces that involve ionic interactions in Gai (E28, D315 and D350) missing in Gao (Figure S9).

Examination of functionally biased ligands in previous studies attributed a major significance to His393 in TM6 [21,23], whose distinct rotamer conformations were herein shown to stabilize different conformations of the ligand-binding pocket of D2R (Figure 3). Thus, His393 can act as a switch that connects the behavior of ligands to distinct conformational ensembles on the intracellular side of the receptor. Further MD-simulations with selected ligands and/or G-proteins are therefore necessary to elucidate the impact of His393 on functionally selective signaling on a molecular level.

We exploited the crystal structure of the ternary b2AR-Gas-complex to establish an active-state model of the pharmacologically highly relevant dopaminergic D2 receptor in complex with...
Figure 8. Comparison of the C-terminal parts of G\textsubscript{i}s and G\textsubscript{i}b. The different conformations of the C-termini of G\textsubscript{i}s (light-blue ribbons) and G\textsubscript{i}b (light-green ribbons) within their pockets in \textbeta\textalpha\textgamma AR (dark-blue ribbons) and D\textsubscript{2}R\textsuperscript{downR} (dark-green ribbons), respectively, are shown. Important residues are represented as sticks. doi:10.1371/journal.pone.0067244.g008

the G-protein subunit G\textsubscript{i}s and the endogenous ligand dopamine. Different computational methods including molecular-dynamics simulations and computational alanine-scanning mutagenesis were used to identify distinct hot-spot residues that determine receptor-G-protein selectivity (Figure 4, 6). Additionally, we transferred our results to closely related aminergic GPCRs and found highly conserved amino acids of receptor subtypes preferentially coupling to G\textsubscript{i}s or G\textsubscript{i}b (Figure 7). As structural information for most GPCR-G-protein complexes is still missing, the computational approach described here is of general importance for investigating protein-protein interfaces of ternary complexes and understanding the determinants of functionally selective signaling.

Our computational approach provides firm predictions with respect to amino acids determining selectivity between GPCRs and G-proteins that can now be confirmed experimentally. The impact of water molecules and possible entropic contributions to selective receptor-G-protein coupling were neglected. In the near future, increasing computational power may give the modeling community the opportunity to visualize the activation of a GPCR and its binding to a G-protein in “real time” and to perform such investigations on a higher level of accuracy. A detailed knowledge of the distinct conformational steps involved in receptor activation upon ligand binding and receptor-G-protein coupling will be a prerequisite on the way to fully reveal the secrets of GPCR-signaling.

Materials and Methods

Homology Modeling

We used the crystal structure of the \textbeta\textalpha\textgamma-adrenergic receptor (\textbeta\textalpha\textgamma AR) together with a heterotrimeric G-protein [16] (PDB-ID: 3SN6) as a starting point for our calculations. The coordinates of the \textbeta\textalpha\textgamma AR and the G\textsubscript{i}RAS-part of the G\textsubscript{i}RAS-protein were used as a template to create a homology model of the dopaminergic D\textsubscript{2} receptor (D\textsubscript{2}R) in complex with a G\textsubscript{i}R-protein. We omitted the \textbeta\gamma-subunit because it has been shown that the (acylated) \textalpha-subunit is sufficient to interact with a G-protein coupled receptor [46]. Three amino acids in the extracellular loop 2 (EL2) of \textbeta\textalpha\textgamma AR that are not resolved in the crystal structure were taken from a nanobody-stabilized active-state structure of the \textbeta\textalpha\textgamma AR [15] (PDB-ID: 3P0G), the 16 residues missing in the area around \textalpha4 of G\textsubscript{\textbeta\textgamma}RAS were modeled manually according to the structure of the GTP\textgamma-bound G\textsubscript{\textbeta\textgamma}R-protein [47] (PDB-ID: 1AZT). The amino-acid sequences for GPCRs and G-proteins were retrieved from the SWISS-PROT database [48]. \textbeta\textalpha\textgamma AR and D\textsubscript{2}R sequences (together with 16 additional sequences of family A GPCRs) as well as G\textsubscript{i}R and G\textsubscript{\textbeta\textgamma}R sequences (together with 4 additional G\textsubscript{\textbeta\textgamma}R protein sequences) were aligned using ChalitaX [49] (Gonnet series matrix with a gap open penalty of 10 and a gap extension penalty of 0.2). The initial sequence alignment was manually refined where necessary by means of BioEdit [50] in order to achieve a perfect alignment of the highly conserved amino acids. Absent parts of the \textbeta\textalpha\textgamma-G\textsubscript{i}R-complex structure (i.e. intracellular loop 3 of \textbeta\textalpha\textgamma AR and G\textsubscript{\textbeta\textgamma}R-\textalphaH of G\textsubscript{\textbeta\textgamma}R) were omitted in the alignment. It has been shown experimentally that removing the bulk of IL3 within the \textbeta\textalpha\textgamma AR does not prevent the receptor from coupling to its G-protein [51]. In addition, constructs of the muscarinic receptors M2 and M3, in which the central region of IL3 (more than 100 amino acids) was omitted, were still able to bind their G-proteins selectively and with near wild type efficacy [30,38]. Therefore, we assume that the truncated D\textsubscript{2}R used in our investigations is still selectively coupled to its G-protein [51].

Based on the final alignment and the \textbeta\textalpha\textgamma-G\textsubscript{i}R-complex structure as a template, we created 50 models of the D2R-G\textsubscript{\textbeta\textgamma}RAS-complex using MODELLER 9v4 [52]. We observed two different rotamer conformations of residue His39\textgamma6\textgamma5 in the D2R-protein with the side chain of His39\textgamma6\textgamma5 pointing to the extracellular and intracellular part of the receptor, respectively.

We selected two models of the D2R-G\textsubscript{\textbeta\textgamma}RAS-complex (referred to as D\textsubscript{2}R\textsuperscript{upR} and D\textsubscript{2}R\textsuperscript{downR}) for further investigation. The models showed the canonical disulfide bond between residue Cys107\textgamma3.25 and the observation that the highly homologous dopaminergic D3 receptor exhibits a second disulfide bond in an equivalent position [53].

Structure Refinement and Modification

The two D2R-G\textsubscript{\textbeta\textgamma}RAS-complexes were submitted to energy minimization in order to remove bad van der Waals contacts of the amino-acid side chains. The SANDER classic module of the AMBER10 program package was used [54]. We applied 500 steps of steepest descent minimization, followed by 4,500 steps of conjugate gradient minimization. The minimization steps were carried out in a water box with periodic boundary conditions and a nonbonded cutoff of 10.0 \textAA. The all-atom force field ff99SB [55] was used.

In order to avoid unnecessarily high flexibility during the simulation process caused by open ends in the G\textsubscript{i}s part of the complexes, we completed the structure of G\textsubscript{i}s by modeling the missing helical part of G\textsubscript{i}s (G\textsubscript{i}s-\textalphaH) manually according to the crystal structure of a GDP-bound heterotrimeric G\textsubscript{\textbeta\textgamma}R-\textbeta\gamma2-protein [56] (PDB-ID: 1GP2) and submitted both complexes to energy minimization [see procedure described above]. Dopamine was manually docked into D\textsubscript{2}R\textsuperscript{upR-G\textsubscript{\textbeta\textgamma}R} and D\textsubscript{2}R\textsuperscript{downR-G\textsubscript{\textbeta\textgamma}R} to obtain agonist-bound ternary GPCR-G-protein systems. The two nucleotide-free ternary D2R-complexes were minimized with SANDER according to the procedure described above using the general AMBER force field (GAFF) [57] for the dopamine atoms and ff99SB for protein residues. Parameters for dopamine were assigned using antechamber [54] and charges were calculated
using Gaussian 09 [58] at the HF/6-31(d,p) level and the RESP procedure according to the literature [59]. A formal charge of +1 was defined for dopamine.

The structural information for the majority of the missing G\textsubscript{\alpha}AH in the β2AR-G\textsubscript{\alpha}RAS-complex was taken from the crystal structure of the GTP\textsubscript{\beta}\textsubscript{\gamma}-bound G\textsubscript{\alpha}R-protein (PDB-ID: 1AZT). A small loop of G\textsubscript{\alpha}i, that connects the G\textsubscript{\alpha}RAS- and the G\textsubscript{\alpha}AH-subunits, the \(\alpha\)1-\(\alpha\)2-loop, still not resolved, was modeled manually according to the crystal structure of 1GP2 (residues I55 to K70). Non-conserved residues between G\textsubscript{\alpha}i and G\textsubscript{\alpha}s were mutated by means of PyMOL [60]. The final structure, comprised of the agonist BI167107, β2AR and the nucleotide-free G\textsubscript{\alpha}s, was submitted to energy minimization using the procedure described above for the D2R-G\textsubscript{\alpha}RAS-systems. Parameters and charges for the ligand BI167107 were used as described above and a formal charge of +1 was attributed to BI167107.

Preparation of the Simulation Systems

Parameter topology and coordinate files for the minimized complexes (BI167107-β2AR-G\textsubscript{\alpha}s, dopamine-D2\textsuperscript{\beta}\textsubscript{\gamma}\textsuperscript{R}-G\textsubscript{\alpha}s, and dopamine-D2\textsuperscript{Down}\textsuperscript{R}-G\textsubscript{\alpha}s) were build up using the tleap module of AMBER10 and subsequently converted into GROMACS input files [61,62].

Each complex was inserted into a dioleoylphosphatidylcholine (DOPC) membrane according to a procedure applied successfully earlier [9].

A pre-equilibrated system bearing a hydrated membrane with 72 DOPC lipids [63] was used. This system had to be enlarged in the x, y and z dimensions in order to surround the ternary complexes fully using a method described earlier [9]. The resulting membrane contained 460 DOPC lipids. According to the density profiles of the membrane, the distribution of all components was confirmed to be as expected without water invading the lipophilic parts of the membrane (Figure S3).

The charges of the simulation systems were neutralized by adding 3 sodium and 8 chloride atoms to the β2AR and the D2R complexes, respectively. In total, the BI167107-β2AR-G\textsubscript{\alpha}s system consisted of 223,264 atoms (659 amino acids, 49,661 water molecules), the dopamine-D2\textsuperscript{\beta}\textsubscript{\gamma}\textsuperscript{R}-G\textsubscript{\alpha}s system of 227,641 atoms (624 amino acids, 51,333 water molecules) and the dopamine-D2\textsuperscript{Down}\textsuperscript{R}-G\textsubscript{\alpha}s system of 224,760 atoms (624 amino acids, 50,188 water molecules).

Membrane Simulations

For all simulations, GAFF was used for the ligands and the DOPC molecules and the force field ff99SB for the protein residues. The SPC/E water model [64] was applied.

After insertion into the prepared membrane, the simulation systems were submitted to energy minimization, equilibration (100 ns) and production molecular-dynamics simulation runs (1 μs) at 310 K using the GROMACS simulation package [61,62] as described earlier [9]. Initial gaps between GPCRs and DOPC-lipids were shown close perfectly during the equilibration (Figure S2).

Throughout the productive simulations a force of 1.0 kcal mol\(^{-1}\) Å\(^{-2}\) was applied to the N-terminal part of the G-protein’s \(\alpha\)N-helix. In vivo, the \(\alpha\)N-helix is anchored to the membrane via acylation with fatty acids and further stabilized by the βγ-subunit when the G-protein is nucleotide-free or bound to GDP [46,65]. The aim of the applied force is to avoid spurious conformations caused by the high flexibility of the \(\alpha\)N-helix in the absence of both the βγ-subunit and the stabilizing acylations because the amino acids that could potentially be acylated are not resolved in the crystal structure of the ternary complex (PDB-ID: 3SN6).

Data Analysis

The analysis of the trajectories was performed with the PTRAJ module of AMBER10. Calculation of the binding free energies and computational alanine scanning mutagenesis was accomplished using the script MMPBSA.py [33]. As our simulations systems are very large, water molecules had to be deleted from the trajectories before analyzing the data in order to reduce the computational demand of the calculations. Therefore, we cannot preclude the existence of further interactions between GPCRs and G-proteins mediated by water molecules. At least for the interactions revealed by our contact analysis, the interacting amino acids are close enough to each other to form stable interactions, even without water molecules.

Figures were prepared using PyMOL [60].

Supporting Information

Figure S1 Conformational changes in the active-state models of the D2R-G\textsubscript{\alpha}s-complex. The backbone atoms of GPCRs and G-proteins are shown as ribbons, whereas residue R380 and the nucleotides of the G-proteins are represented as sticks and spheres, respectively. Red arrows denote major helical movements upon receptor activation. (A) Intracellular view of the superposition of active-state models of D2\textsuperscript{Down}\textsuperscript{R} (green) and D2\textsuperscript{R} (dark-red) and the crystal structures of D3R (PDB-ID 3PBL, grey) and β2AR in complex with different binding partners (violet: carazolol, PDB-ID 2RH1; dark-blue: FAUC50, PDB-ID 3PDS; blue: BI167107 and the G\textsubscript{\alpha}i protein, PDB-ID 3SN6). (B) Side view of one part of the receptor-G-protein interface of D2\textsuperscript{Down}\textsuperscript{R}-G\textsubscript{\alpha}s (green), D2\textsuperscript{R}-G\textsubscript{\alpha}s (dark-red) and β2AR-G\textsubscript{\alpha}s (blue). The crystal structures of G\textsubscript{\alpha}s in complex with GDP (PDB-ID 1GP2, orange) and of G\textsubscript{\alpha}s together with GTP\beta\gamma (PDB-ID 1AZT, yellow) are aligned on the G-proteins components of the ternary complexes. (TIFF)

Figure S2 Equilibration of the simulation systems. (A) The β2AR-system (blue ribbons) is shown from the top after insertion into the DOPC-bilayer (grey sticks), but before equilibration steps were performed. (B) After equilibration, the gaps between the receptor and the membrane appeared to be perfectly closed. (C) A side view on the β2AR-G\textsubscript{\alpha}s simulation system is provided. β2AR and G\textsubscript{\alpha}s are shown as blue ribs. The ligand BI167107 is represented as orange spheres, and the DOPC-molecules as grey sticks. Water molecules are removed for clarity. (TIFF)

Figure S3 Density profiles of the simulation systems. The partial density profiles of individual components of the simulation systems are shown for the simulation time steps 0–100 ns (first 100 ns) and 900–1000 ns (last 100 ns). (TIFF)

Figure S4 RMS-deviations within the MD simulations. (A) The RMS-deviations for the individual components of the β2AR-G\textsubscript{\alpha}s system are shown. Values for the ligand BI167107, β2AR and G\textsubscript{\alpha}s are given in yellow, dark-blue and light-blue, respectively. (B) The RMS-deviations for the individual components of the D2\textsuperscript{Down}\textsuperscript{R}-G\textsubscript{\alpha}s system are shown. Values for the ligand dopamine, D2\textsuperscript{Down}\textsuperscript{R} and G\textsubscript{\alpha}s are given in orange, dark-green and light-green, respectively. (C) The RMS-deviations for the individual components of the D2\textsuperscript{R}-G\textsubscript{\alpha}s system are shown. Values for the ligand dopamine, D2\textsuperscript{R} and G\textsubscript{\alpha}s are given in orange, dark-red and light-red, respectively. The ligands and the receptors are fitted on the C\textalpha-atoms of the receptors, whereas the G-proteins are fitted on the C\textbeta-atoms of the G-proteins. Grey rectangles
Figure S5 Atomic fluctuations within the MD simulations. The atomic fluctuations for the Cα-atoms of the β2AR-Gαi-complex (A), the D2DownR-Gαi-complex (B) and the D2UpR-Gαi-complex (C) are given in blue, green and red, respectively. The thickness of the lines indicate different fitting procedures (on Cα-atoms): the thick lines for receptors and G-proteins point to a fit on the receptors and the G-proteins, respectively, whereas the thin lines mean that the G-proteins were fitted on the receptor moieties. (TIF)

Figure S6 Conformational changes of Gαi within the D2DownR-Gαi-simulations. (A, B) The D2DownR- and the D2UpR-Gαi-complexes are shown as green and red ribbons, respectively. Residues R227 and D315 are represented as sticks. (C) The distance between the atoms CZ of R227 and CG of D315 is depicted throughout the MD simulations (green: D2DownR-Gαi, red: D2UpR-Gαi). (TIFF)

Figure S7 Distances between receptors and G-proteins within the MD simulations. (A) The distances between the centers of mass of β2AR and the whole Gαi, and β2AR and the C-terminus of Gαi are shown in dark-blue and light-blue, respectively. (B) The distances between the centers of mass of D2DownR and the whole Gαi and D2DownR and the C-terminus of Gαi are shown in dark-green and light-green, respectively. (C) The distances between the centers of mass of D2UpR and the whole Gαi and D2UpR and the C-terminus of Gαi are shown in dark-red and light-red, respectively. (TIFF)

Figure S8 Free energies of binding for the ternary complexes. The free energies of binding for the β2AR-Gαi system (A), for the D2DownR-Gαi system (B) and for the D2UpR-Gαi system (C) are shown. Here, the free energy of binding consists of a molecular mechanics energy term (internal energy of bonds, angles and dihedrals), the polar contribution and the nonpolar contribution of the solvation free energy (polar contribution calculated using the Generalized Born equation and the nonpolar contribution using the molecular solvent-accessible surface area). The curves exhibit a best fit line with a positive gradient for (A) and (B) (0.012 and 0.021 for the D2 DownR-Gαi, respectively), and a negative gradient for curve (C) (−0.021 for the D2 UpR-Gαi-system). As these gradients are very small, we expect that the values will converge to zero for longer simulation times. (TIF)

Figure S9 Alignment of contact areas of chosen Gαi-subunits. Amino acids within the Gαi and Gαo sequences forming stable contacts to receptor residues are highlighted with a blue and green background, respectively (according to Figure 4). Red backgrounds point to sequence differences between Gαi and Gαo subunits. Red letters indicate residues involved in ion interactions. (TIF)

Table S1 Amino-acid contacts within the β2AR-Gαi-simulation. The occurrence for each amino-acid contact throughout the MD simulation is shown in the grey columns. (DOC)

Table S2 Amino-acid contacts within the D2DownR-Gαi-simulation. The occurrence for each amino-acid contact throughout the MD simulation is shown in the grey columns. (DOC)

Table S3 Amino-acid contacts within the D2UpR-Gαi-simulation. The occurrence for each amino-acid contact throughout the MD simulation is shown in the grey columns. (DOC)

Table S4 Results of the computational alanine scanning for the receptors and the G-proteins. aa refers to the amino acids mutated to alanine. ΔAG-values are provided in the format ‘value ± standard deviation’. The left column shows the regions within the GPCRs and the G-proteins, to which the mutated amino acids belong. (DOC)

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Author Contributions

Conceived and designed the experiments: RCK HL TC PG. Performed the experiments: RCK. Analyzed the data: RCK. Wrote the paper: RCK PG.

References

1. Fredriksson R, Lagerstrom MC, Lundin LG, Schioth HB (2003) The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogue groups, and fingerprints. Mol Pharmacol 63: 1256–1272.
2. Lagerstrom MC, Schioth HB (2008) Structural diversity of G protein-coupled receptors and significance for drug discovery. Nat Rev Drug Discov 7: 339–357.
3. Ahuja S, Smith SO (2009) Multiple switches in G protein-coupled receptor activation. Trends Pharmacol Sci 30: 494–502.
4. Nygaard R, Fimrumer TM, Holst B, Rosenkilde MM, Schwartz TW (2009) Ligand binding and micro-switches in 7TM receptor structures. Trends Pharmacol Sci 30: 249–259.
5. Trzaskowski B, Latke D, Yuan S, Ghooshdistader U, Debinski A, et al. (2012) Action of molecular switches in GPCRs-theoretical and experimental studies. Curr Med Chem 19: 1090–1109.
6. Drot RO, Arlow DH, Maragakis P, Mildorf TJ, Pan AC, et al. (2011) Activation mechanism of the beta2-adrenergic receptor. Proceedings of the National Academy of Sciences of the United States of America 108: 18684–18689.
7. Venkatakrishnan AJ, Deupi X, Lebon G, Tate CG, Schertler GF, et al. (2013) Molecular signatures of G-protein-coupled receptors. Nature 494: 185–194.
8. Deupi X, Standfuss J, Schertler G (2012) Conserved activation pathways in G-protein-coupled receptors. Biochem Soc Trans 40: 303–308.
9. Goetz A, Lanig H, Gmeiner P, Clark T (2011) Molecular Dynamics Simulations of the Effect of the G-Protein and Diffusible Ligands on the β2-Adrenergic Receptor. Journal of molecular biology.
10. De Lean A, Stadel JM, Lefkowitz RJ (1980) A ternary complex model explains the agonist-specific binding properties of the adenylate cyclase-coupled beta-adrenergic receptor. J Biol Chem 255: 7108–7117.
11. Karrich V, Chenoweth V, Stevens RC (2012) Structure-Function of the G-Protein-Coupled Receptor Superfamily. Annu Rev Pharmacol Toxicol.
12. Rasmussen SG, Choi HJ, Rosenbaum DM, Kohki TA, Thian FS, et al. (2007) Crystal structure of the human beta2 adrenergic G-protein-coupled receptor. Nature 450: 383–387.
13. Chenoweth V, Rosenbaum DM, Hansen MA, Rasmussen SG, Thian FS, et al. (2007) High-resolution crystal structure of an engineered human beta2-adrenergic G-protein-coupled receptor. Science 318: 1258–1265.
14. Rosenbaum DM, Zhang C, Lyons JA, Hoff R, Aragao D, et al. (2011) Structure and function of an irreversible agonist-beta(2) adrenoceptor complex. Nature 469: 236–240.
15. Rasmussen SG, Choi HJ, Fung JJ, Pardon E, Cauarosa P, et al. (2011) Structure of a nanobody-stabilized active state of the beta[2] adrenoceptor. Nature 469: 175–180.
