A structural basis for how ligand binding site changes can allosterically regulate GPCR signaling and engender functional selectivity

Marta Sanchez-Soto¹, Ravi Kumar Verma², Blair K.A. Willette¹, Elizabeth C. Gonyé¹, Annah M. Moore¹, Amy E. Moritz¹, Comfort A. Boateng³, Hideaki Yano², R. Benjamin Free¹, Lei Shi²*, David R. Sibley¹*

Signaling bias is the propensity for some agonists to preferentially stimulate G protein–coupled receptor (GPCR) signaling through one intracellular pathway versus another. We previously identified a G protein–biased agonist of the D₂ dopamine receptor (D₂R) that results in impaired β-arrestin recruitment. This signaling bias was predicted to arise from unique interactions of the ligand with a hydrophobic pocket at the interface of the second extracellular loop and fifth transmembrane segment of the D₂R. Here, we showed that residue Phe189 within this pocket (position 5.38 using Ballesteros-Weinstein numbering) functions as a microswitch for regulating receptor interactions with β-arrestin. This residue is relatively conserved among class A GPCRs, and analogous mutations within other GPCRs similarly impaired β-arrestin recruitment while maintaining G protein signaling. To investigate the mechanism of this signaling bias, we used an active-state structure of the β₂-adrenergic receptor (β₂R) to build β₂R-WT and β₂R-Y199⁵.38A models in complex with the full β₂R agonist BI-167107 for molecular dynamics simulations. These analyses identified conformational rearrangements in β₂R-Y199⁵.38A that propagated from the extracellular ligand binding site to the intracellular surface, resulting in a modified orientation of the second extracellular loop in β₂R-Y199⁵.38A, which is predicted to affect its interactions with β-arrestin. Our findings provide a structural basis for how ligand binding site alterations can allosterically affect GPCR-transducer interactions and result in biased signaling.

INTRODUCTION

G protein–coupled receptors (GPCRs) represent the largest family of cellular receptors in mammals and are critical drug targets accounting for about one-third of all U.S. Food And Drug Administration–approved drugs (1). These receptor proteins regulate multiple physiological processes by transducing extracellular stimuli, such as neurotransmitters, hormones, peptides, or light, into intracellular signals through activating both G protein–dependent and G protein–independent pathways, leading to second messenger generation and downstream signaling events. G protein–independent pathways are primarily mediated by β-arrestin proteins (2–4), which were originally identified as mediators of agonist-induced desensitization and receptor endocytosis but were subsequently determined to also function as multivalent scaffolding proteins that orchestrate various intracellular signaling pathways (5). Although endogenous agonists promote GPCR signaling through the activation of both G proteins and β-arrestins, these events often occur in a temporally separate fashion (6–8). In contrast, some synthetic agonists have been described to preferentially activate discrete signaling pathways versus others, a phenomenon known as functional selectivity or biased signaling (9–13). The therapeutic potential of biased signaling is high because drugs that selectively modulate clinically relevant pathways, without affecting other signaling events, may exhibit fewer side effects (14, 15). Although the molecular mechanisms underlying biased signaling are not known with certainty, a leading hypothesis is that GPCRs can adopt distinct active conformational states that are selectively stabilized by different signaling-biased ligands (16–20). A detailed understanding of the structural determinants underlying agonist-specific signaling states of GPCRs should allow for the rational design of novel functionally selective agents (13).

Biased signaling can occur not only in response to ligands but also from mutations in GPCRs, resulting in restricted signaling to specific pathways. For instance, Caron and colleagues have used the evolutionary trace (ET) method (21) to identify D₂ dopamine receptor (D₂R) mutants that selectively signal through either G proteins or β-arrestins (22, 23). Similarly, Schönegge et al. (24) used the same ET method to identify signaling-biased mutants of the β₂-adrenergic receptor (β₂R) that were selectively impaired in either Gₛ- or β-arrestin–but not Gₛ–mediated signaling. Conversely, Donthamsetti et al. (25) reported a double mutant of the D₂R that could robustly recruit β-arrestin but that was devoid of G protein–mediated signaling. β-Arrestin–biased mutants of the M₃ muscarinic receptor have also been developed for use in the “designer receptors exclusively activated by designer drugs” (DREADD) technology (26). None of the mutations described in these previous studies were within, or close to, the ligand binding sites, but rather were situated near the intracellular surface of the receptors. Further, the previously studied mutations were not investigated using related GPCRs, and thus, their generalizability is unclear.

We previously described a G protein–biased D₂R agonist, MLS1547, that is efficacious for G protein–mediated signaling but relatively ineffective in β-arrestin recruitment (27, 28). Structure-activity relationship analyses using MLS1547 and its analogs led to a pharmacophore model in the context of receptor structure to explain the biased signaling properties of this compound. This involved the interaction of the ligand with a hydrophobic pocket composed of residues Ile184,

Phe189, and Val190 within the fifth transmembrane region (TM5) and second extracellular loop (EL2) of the D2R. Here, we identify residue Phe189 in the D2R [position 5.38 using the Ballesteros-Weinstein numbering system (29)] as a microswitch that regulates the active state for recruiting β-arrestin. Our findings showed that such a switch exists not only for the D2R but also for several related GPCRs, including the β2R. Molecular dynamics (MD) simulations using an active-state structure of the β2R (30) revealed that mutation of residue 5.38 resulted in conformational rearrangements that propagate from the extracellular ligand binding site to the intracellular surface, leading to an altered orientation of intracellular loop 2 (IL2), which is predicted to affect β-arrestin interactions, thus conferring biased signaling.

RESULTS

Investigation of structural elements supporting signaling bias by the D2R agonist MLS1547

We previously suggested (27, 28) that the G protein–biased agonist MLS1547 uniquely interacts with a hydrophobic pocket of the D2R composed of residues Ile184EL2, Phe1895.38, and Val1905.39 at the junction between the extracellular tip of TM5 and EL2 of the D2R (Fig. 1A) (31). Detailed structure-activity analyses showed that congeneric compounds of MLS1547 lacking a hydrophobic moiety oriented toward this pocket exhibit more balanced G protein– and β-arrestin–mediated signaling (27, 28), supporting the idea that ligand interactions with this pocket confer signaling bias. To further investigate the role of this binding pocket in D2R signaling, we created alanine mutations of the residues enclosing this pocket (I184EL2 A, F1895.38 A, and V1905.39 A). We found that the singly mutated receptors were expressed at a comparable degree as the wild-type D2R (D2R-WT) in cells also expressing G protein (fig. S1A) and β-arrestin (fig. S1B) assay components. The I184EL2 A or V1905.39 A mutations decreased the potency and maximum response of MLS1547 for G protein activation, as measured using a bioluminescence resonance energy transfer (BRET)–based assay with biosensors fused to the α and γ subunits of Go, an endogenous transducer of the D2R (Fig. 1B and table S1) (32). The effects of the F1895.38 A mutation were more pronounced, resulting in a complete loss of MLS1547’s ability to activate Go (Fig. 1B and table S1). However, MLS1547 could still interact with the D2R-F1895.38 A, as demonstrated by its ability to functionally antagonize dopamine signaling (Fig. 1C) and compete for radioligand binding (table S2). These results suggest that the primary effect of the F1895.38 A mutation is the elimination of MLS1547 efficacy for G protein activation. These results highlight the importance of this hydrophobic pocket and, in particular, identify Phe1895.38 in TM5 as a pivotal residue in regulating the biased signaling of MLS1547 through the D2R.

Identification of a G protein signaling–biased mutant D2R

We next evaluated whether perturbation of this hydrophobic pocket affected the signaling properties of dopamine through the D2R. Radioligand binding assays revealed that the I184EL2 A, F1895.38 A, and V1905.39 A mutant receptors exhibited a 3- to 10-fold reduction in the affinity of dopamine (Fig. 1D and table S2). For the I184EL2 A and V1905.39 A mutants, the potency for dopamine activation of Go was reduced without changes in the maximum response (Fig. 1E and table S1). Similarly, BRET-based analysis of β-arrestin recruitment to the I184EL2 A and V1905.39 A mutants showed that the potency of dopamine was reduced without a change in Emax (Fig. 1F and table S1). For the D2R-F1895.38 A mutant, the potency of dopamine was reduced for Go activation, similar to the I184EL2 A and V1905.39 A mutants, whereas the maximum response was comparable to that of the WT receptor (Fig. 2A and table S1). Similar results were observed using a different G protein–mediated assay measuring D2R-mediated inhibition of forskolin-stimulated cyclic adenosine monophosphate

Fig. 1. Investigation of structural elements supporting G protein–biased signaling by the D2R. (A) Pharmacophore model for MLS1547 interactions with the D2R [modified from (27)]. (B) The D2R-WT or the indicated D2R mutants were expressed in HEK293 cells with Go–α, Rluc8, β1, and γ2-mVenus. The cells were stimulated with MLS1547 and assessed for G protein activation by BRET. (C) HEK293 cells expressing D2R-F1895.38 A, Gox1–Rluc8, β1, and γ2-mVenus were incubated with 13 μM (EC50) dopamine and the indicated concentrations of either sulpiride or MLS1547 and assessed for G protein activation by BRET. (D) Membrane preparations from HEK293 cells expressing either D2R-WT or D2R-I184EL2 A, V1905.39 A, or F1895.38 A were incubated with the indicated concentrations of dopamine and [3H]methylspiperone. Data are expressed as a percentage of the specific binding and fit using nonlinear regression analyses (table S2). (E) HEK293 cells described in (B) were stimulated with dopamine and assayed for G protein activation. (F) The D2R-WT and indicated mutant receptors were fused to Rluc8 and expressed with β-arrestin2–mVenus and GRK2 in HEK293 cells. Dopamine-stimulated β-arrestin recruitment was assessed by BRET. Functional data are expressed as a percentage of the maximum dopamine or MLS1547 responses for D2R-WT (% control). Data in (B) to (F) represent the mean ± SEM values of three to five independent experiments performed in technical triplicate. Average EC50 and Emax values for functional assays are displayed in table S1.


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Fig. 2. The F189S38A mutation confers G protein signaling bias in the D2R. (A) HEK293 cells transiently expressing either D2R-WT or D2R-F189S38A with Go11-Rluc8, β1, and γ2-mVenus were stimulated with dopamine and assayed for G protein activation by BRET. Average EC50 and Emax values are displayed in table S1. (B) HEK293 cells transiently expressing D2R-WT or D2R-F189S38A with the CAMYEL biosensor were assayed for inhibition of forskolin-stimulated cAMP production. Average EC50 and Emax values are displayed in table S3. (C) The D2R-WT and F189S38A receptors were fused to Rluc8 and expressed in HEK293 cells with β-arrestin2–mVenus and GRK2. Dopamine-stimulated β-arrestin recruitment was assessed by BRET. Average EC50 and Emax values are displayed in table S1. (D) D2R-WT or D2R-F189S38A were fused to a segment of β-galactosidase and expressed in CHO cells with β-arrestin2 fused to a complementing segment of β-galactosidase. Dopamine-stimulated complementation of β-galactosidase was measured. Average EC50 and Emax values are shown in table S3. (E and F) Molecular proximity between D2R-WT or D2R-F189S38A and β-arrestin2 was detected with titration experiments performed in HEK293 cells. Cells expressing a fixed amount of D2R-WT–Rluc8 or D2R-F189S38A–Rluc8 and increasing amounts of β-arrestin2–mVenus were incubated in the presence or absence of 10 μM quinpirole. β-Arrestin recruitment was assessed by BRET. X axes represent the ratio between the fluorescence emitted by β-arrestin2–mVenus and the luminescence emitted by D2R-WT or D2R-F189S38A–Rluc8. Y axes represent the BRET ratio. (G and H) The D2R-WT and indicated mutant receptors fused to Rluc8 were expressed in HEK293 cells with β-arrestin2–mVenus and GRK2. (G) Dopamine- or (H) pramipexole-stimulated β-arrestin recruitment was assessed by BRET. Average EC50 and Emax values are displayed in table S5. All functional data are expressed as percentage of the maximum response observed for D2R-WT. Data points in (A) to (H) represent mean ± SEM of 3 to 14 independent experiments performed in technical triplicate.
The D2R-F189 5.38A mutant exhibited a decrease in dopamine potency for inhibiting cellular cAMP levels, although the maximal response for this response was comparable to that for D2R-WT (Fig. 2B and table S3). Thus, although the D2R-F189 5.38A displayed reduced potency for dopamine stimulation of G protein–mediated signaling, the maximum response appeared to be unchanged. In contrast, dopamine was unable to stimulate β-arrestin recruitment for the D2R-F189 5.38A, as assessed by either the β-arrestin BRET assay (Fig. 2C) or an enzyme complementation assay that measures the recruitment of β-arrestin to the receptor (Fig. 2D). Similar results were observed for other full agonists such that β-arrestin recruitment was either lost (for pramipexole and quinpirole) or greatly diminished (for rotigotine and apomorphine) with the D2R-F189 5.38A mutant, whereas G protein activation was largely maintained with variable decreases in potency (fig. S2, A to H, and table S4). Calculation of bias factors that take into account effects on both median effective concentration (EC50) and Emax (33) for rotigotine and apomorphine, which exhibit residual β-arrestin recruitment in the D2R-F189 5.38A mutant, confirmed their G protein–mediated signaling bias (table S4).

To further confirm the diminished ability of agonists to recruit β-arrestin to the D2R-F189 5.38A mutant, we performed a BRET saturation assay (Fig. 2, E and F) in which the expression of the BRET donor (D2R-RLuc8) was held constant, whereas that of the BRET acceptor (β-arrestin–mVenus) was increased, thus altering the donor/acceptor ratios. In the presence of the full D2R agonist quinpirole, the BRET signal was saturated with increasing β-arrestin–mVenus when the D2R-WT was used (Fig. 2E). In contrast, using the D2R-F189 5.38A mutant (Fig. 2F), quinpirole did not produce a saturable BRET signal, confirming the inability of this mutant to recruit β-arrestin in the presence of agonist.

One question concerning the differential effects of the D2R-F189 5.38A mutation on G protein– and β-arrestin–mediated signaling was the degree of amplification in the G protein–mediated assays compared with the β-arrestin assays, which lack amplification. If the G protein–mediated assays were extremely amplified, then the F189 5.38A mutation might negatively affect signaling efficacy without an observable effect on the maximum response in the assay. With respect to the D2R-WT, the potency of dopamine was 4- to 15-fold greater for stimulating G protein–mediated signaling compared with β-arrestin recruitment, suggesting some degree of amplification (tables S1 and S3). However, to assess this more directly, we compared the effects of a partial agonist of the D2R in the two assays. If the G protein–mediated assay was extremely amplified, then the relative Emax for the partial agonist should be much greater than that observed in the β-arrestin recruitment assay. The D2R partial agonist CAB02-110 (compound 11) (34) was about ninefold more potent in the G protein assay; however, its Emax (compared with dopamine) was only marginally higher compared with that in the β-arrestin assay (68% compared with 58%, respectively) (fig. S3). As observed previously (tables S1 and S3), dopamine was 15-fold more potent in the G protein signaling assay (fig. S3). These results suggest that, although there was some degree of amplification in the G protein–mediated assay, it was not sufficiently high so as to obscure interpretation of the differential effects of the F189 5.38A mutation on the two signaling arms of the D2R.

We further assessed D2R-mediated β-arrestin recruitment in response to either dopamine (Fig. 2G) or the D2R agonist pramipexole (Fig. 2H) in mutants in which Phe189 5.38 was substituted using amino acid residues with different physicochemical properties. The only amino acid substitution that did not negatively affect agonist-stimulated β-arrestin recruitment was the replacement of phenylalanine with tyrosine, a structurally similar aromatic amino acid (Fig. 2, G and H, and table S5). Together, these results indicate that the D2R-F189 5.38A mutant is selectively biased toward G protein–mediated signaling and deficient with respect to β-arrestin recruitment.

**Impairment of agonist-stimulated internalization of the D2R-F189 5.38A mutant**

A major function of β-arrestin recruitment is to initiate endocytosis of GPCRs into clathrin-coated pits, thereby removing them from the cell surface (3, 35, 36). Previously, we showed that β-arrestin2 mediates agonist-stimulated D2R internalization in neurons (37). To evaluate the internalization of the G protein–biased D2R-F189 5.38A, we used [3H]sulpiride, a D2R antagonist that only labels cell surface receptors in intact cell binding assays due to its hydrophilicity and inability to cross the cell membrane (38, 39). Pretreatment with dopamine significantly decreased cell surface D2R-WT (Fig. 3A), as we have previously described (28, 38, 39). In contrast, dopamine pretreatment did not affect the cell surface binding of [3H]sulpiride in cells expressing the D2R-F189 5.38A, indicating a lack of agonist-induced receptor internalization (Fig. 3B). We next measured constitutive BRET between the D2R and the plasma membrane–localized tyrosine kinase Lyn (40), which is decreased by agonist-induced internalization of the D2R. Treatment with dopamine dose-dependently reduced constitutive D2R-Lyn BRET in cells expressing the D2R-WT but not in those expressing the D2R-F189 5.38A mutant. Together, these results suggest that impairment of β-arrestin recruitment in the D2R-F189 5.38A functionally affects β-arrestin–mediated downstream signaling processes as demonstrated by impaired receptor internalization.

Because the agonists rotigotine and apomorphine exhibit a very low but measurable level of β-arrestin recruitment to the D2R-F189 5.38A mutant (fig. S2, E and G, and table S4), we wondered whether these compounds would promote internalization of the mutant receptor to a corresponding low degree. Rotigotine and apomorphine stimulated maximal internalization of the D2R-WT but did not promote significant internalization of the D2R-F189 5.38A (fig. S4, A and B). These results suggest that, although β-arrestin is partially recruited to the mutant receptor in response to rotigotine and apomorphine, the resulting β-arrestin–D2R-F189 5.38A interactions are no longer sufficient to promote receptor internalization.

**Functional conservation of residue 5.38 in related GPCRs**

Given the importance of Phe189 5.38 in the D2R for determining signaling bias, we investigated the conservation of this and nearby residues among related GPCRs. An alignment of the residues surrounding the EL2-TM5 hydrophobic pocket revealed that residue 5.38 is relatively conserved among related GPCRs. An alignment of the residues surrounding the EL2-TM5 hydrophobic pocket revealed that residue 5.38 is relatively conserved among related GPCRs. Among all 286 human nonolfactory class A GPCRs (Table 1), 88 (31%) have Tyr, whereas 42 (15%) contain Phe at position 5.38 (for rotigotine and apomorphine) with the D2R-F189 5.38A mutant, indicating a lack of agonist-induced receptor internalization (Fig. 3B). We next measured constitutive BRET between the D2R and the plasma membrane–localized tyrosine kinase Lyn (40), which is decreased by agonist-induced internalization of the D2R. Treatment with dopamine dose-dependently reduced constitutive D2R-Lyn BRET in cells expressing the D2R-WT but not in those expressing the D2R-F189 5.38A mutant. Together, these results suggest that impairment of β-arrestin recruitment in the D2R-F189 5.38A functionally affects β-arrestin–mediated downstream signaling processes as demonstrated by impaired receptor internalization.
Fig. 3. The G protein–biased D2R-F1895.38A exhibits impaired agonist-induced internalization. (A and B) HEK293 cells expressing either D2R-WT (A) or D2R-F1895.38A (B) were incubated for 1.5 hours with vehicle or 10 μM dopamine. Surface expression of the receptor was measured with an intact cell binding assay using [3H]sulpiride. Data are representative of three independent experiments. *P < 0.05, unpaired Student’s t test. (C) HEK293 cells transiently expressing either D2R-WT–Rluc8 or D2R-F1895.38A–Rluc8 with LYN-rGFP were treated with increasing concentrations of dopamine for 10 min. The interaction between D2R and LYN was measured by BRET. In the graph, the constitutive basal BRET is defined as 100% control, and maximum dopamine-induced decrease in BRET is defined as 0%. The EC50 for dopamine-induced internalization was 88 ± 19 nM. No measurable internalization was observed with the D2R-F1895.38A. Data in (A) to (C) are mean ± SEM of four independent experiments performed in technical triplicate.


Allosteric propagation of the $\beta2R$-$Y199^{5.38}$ A perturbation to the intracellular surface

To investigate how alterations at position 5.38 of the receptor induce conformational rearrangements that propagate from the extracellular to the intracellular surface resulting in signaling bias, we used the $\beta2R$, for which high-resolution crystal structures of the active state are available, as a model system to study the underlying molecular mechanisms. Specifically, we used the crystal structure of $\beta2R$ in an active conformation [Protein Data Bank (PDB) code 4LDE] (30) for building both $\beta2R$-WT and Y199$^{5.38}$A models in complex with the high-affinity full-agonist BI-167107 and carried out MD simulations (table S7) to detect the perturbation of the Y199$^{5.38}$A mutation on receptor conformation (Fig. 5, A to C). We out MD simulations (table S7) to detect the perturbation of the complex with the high-affinity full-agonist BI-167107 and carried out MD simulations (table S7) to detect the perturbation of the Y199$^{5.38}$A mutation on receptor conformation (Fig. 5, A to C). We

Table 1. Alignment of hydrophobic pocket residues for select GPCRs. The amino acid position in extracellular loop 2 (EL2) is delineated using the nomenclature developed by de Graaf et al. (76). Yellow indicates nonpolar amino acids with an aliphatic group (Ala, Val, Ile, Leu, Met, and Gly); light green indicates hydrophobic amino acids with an aromatic ring (Phe and Tyr); dark green indicates Trp; purple indicates polar amino acids with an uncharged side chain (Ser, Thr, Asn, and Gin); light gray indicates Pro; red indicates negatively charged amino acids (Glu and Asp); and blue indicates positively charged amino acids (Arg, His, and Lys).

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*Amino acid positions in transmembrane (TM) regions are delineated using the Ballesteros and Weinstein nomenclature (29).*
interface from the extracellular surface to the middle of the TM domain (Fig. 6B). The reconfiguration of these interactions in the two complexes affects TM4 and TM5 on their extracellular sides in a similar fashion as we observed when comparing the MD simulations of β2AR-WT and β2R-Y1995.38A and appears to be associated with distinct IL2 conformations on the intracellular side: IL2 is helical in the rhodopsin-arrestin complex and extended in the rhodopsin-Gi complex. Thus, although the residue types are different between the β2AR and rhodopsin within this interface, it may serve as a common mechanistic pathway in propagating the impact of ligand binding from the extracellular to the intracellular side and consequently be differentially affected by functionally selective ligands, resulting in biased signaling. The perturbation of this pathway, such as that by the Y1995.38A mutation in β2R (Fig. 6A and fig. S8) or the F1895.38A mutation in D2R, even from an extracellular location, should have a similar impact.

**DISCUSSION**

It is widely appreciated that GPCRs typically signal through multiple pathways involving different transducers including both G proteins and β-arrestins. As for many GPCRs, biased agonists that selectively...
intracellular signaling properties of the D2R. The phobic pocket may serve as a microswitch to allosterically bias the results suggest that structural features within this extracellular hydrophobic region related with its interaction with a hydrophobic pocket within the D2R consisting of residues Ile184EL2, Phe1895.38, and Val1905.39. These results further support the notion that this D2R pocket in-

dicates that its diminished ability to recruit G protein–

mediated signaling was negated, whereas Gs primarily activates Gs. An alanine mutation of the 5.38 residue (Tyr1995.38) in the D2R resulted in a G protein signaling–biased receptor, which negatively affected agonist-stimulated β-arrestin recruitment while minimally affecting G protein–mediated signaling. Therefore, the observation that D2R-F1895.38A failed to internalize in response to agonist stimulation, a process mediated by β-arrestin2 (37), further supports this conclusion. Together, these results provide evidence that D2R-F1895.38A is biased for G protein–mediated signaling and that Phe1895.38 plays a pivotal role in regulating signaling bias.

A relatively high percentage of class A GPCRs, including catecholamine receptors, have the aromatic amino acids Phe or Tyr in the 5.38 position, suggesting conservation of function for these residues. Tyr was the only amino acid substitution for Phe1895.38 in the D2R that did not negatively affect agonist-stimulated β-arrestin recruitment. Further, substitution of the 5.38 residues in the closely related D3R (Phe1885.38) and D4R (Tyr1925.38) with alanine similarly eliminated agonist-stimulated β-arrestin recruitment while minimally affecting G protein–mediated signaling. Because D2-like receptors couple to the Gi/G0 family, we extended our analyses to β2R, which primarily activates Gi. An alanine mutation of the 5.38 residue (Tyr1995.38) in the β2R resulted in a G protein signaling–biased phenotype in which β-arrestin recruitment was negated, whereas Gi activation was minimally affected. Thus, the retention of G protein signaling seen with the Phe/Tyr5.38 mutants for nonbiased agonists stimulated either G protein– (27, 28, 46–51) or β-arrestin–mediated pathways (13, 48, 52–55) have been discovered for the D2R, although the underlying molecular mechanisms are not well understood. Our previous studies (27, 28) with the G protein–biased agonist MLS1547 indicated that its diminished ability to recruit β-arrestin was correlated with its interaction with a hydrophobic pocket within the D2R consisting of residues Ile184EL2, Phe1895.38, and Val1905.39. These results suggest that structural features within this extracellular hydrophobic pocket may serve as a microswitch to allosterically bias the intracellular signaling properties of the D2R. In support of this idea, McCorry et al. (13) showed that modifying ligands based on the antipsychotic aripiprazole that results in strengthened interactions with D2R residue Ile184EL2 affects their ability to stimulate β-arrestin recruitment. To further test this hypothesis, we evaluated the impact of the single-point mutations Ile184EL2, F1895.38, and V1905.39 on D2R signaling activity. Each of these alterations detrimentally affected the G protein–mediated signaling of MLS1547 with the F1895.38A mutant, resulting in a complete loss of MLS1547 efficacy. These results further support the notion that this D2R pocket includes structural determinants that contribute to both ligand efficacy and signaling bias.

Somewhat different results were obtained with the mutant D2R constructs when we examined signaling in response to the endogenous neutral agonist dopamine. The I184EL2 A and V1905.39 A mutants displayed reduced dopamine-receptor binding affinity and potency for activating both G protein– and β-arrestin–mediated signaling without a loss of functional efficacy for either pathway. In contrast, whereas the F1895.38 A mutant showed reduced dopamine binding affinity and potency for G protein–mediated signaling, as was observed with the other two D2R mutants, its efficacy for stimulating β-arrestin recruitment was completely eliminated. Further, the ability of dopamine to maximally activate G protein–mediated signaling was fully maintained. Because the BRET assays used in these experiments directly measure D2R–β-arrestin interactions, these results suggest that D2R-F1895.38A was impaired in its ability to form an active conformation that recruits and activates β-arrestin. The observation that D2R-F1895.38A failed to internalize in response to agonist stimulation, a process mediated by β-arrestin2 (37), further supports this conclusion. Together, these results provide evidence that D2R-F1895.38A is biased for G protein–mediated signaling and that Phe1895.38 plays a pivotal role in regulating signaling bias.

A relatively high percentage of class A GPCRs, including catecholamine receptors, have the aromatic amino acids Phe or Tyr in the 5.38 position, suggesting conservation of function for these residues. Tyr was the only amino acid substitution for Phe1895.38 in the D2R that did not negatively affect agonist-stimulated β-arrestin recruitment. Further, substitution of the 5.38 residues in the closely related D3R (Phe1885.38) and D4R (Tyr1925.38) with alanine similarly eliminated agonist-stimulated β-arrestin recruitment while minimally affecting G protein–mediated signaling. Because D2-like receptors couple to the Gi/G0 family, we extended our analyses to β2R, which primarily activates Gi. An alanine mutation of the 5.38 residue (Tyr1995.38) in the β2R resulted in a G protein signaling–biased phenotype in which β-arrestin recruitment was negated, whereas Gi activation was minimally affected. Thus, the retention of G protein signaling seen with the Phe/Tyr5.38 mutants for nonbiased agonists...
appears to be independent of G protein coupling preference, whereas β-arrestin recruitment is consistently attenuated.

The nephrogenic diabetes insipidus–associated human polymorphisms at position 5.38 in the V2R result in Cys and His substitutions for Tyr at position 205 (Y2055.38C and Y2055.38H) (41, 42). We found that both of these alterations, as well as a V2055.38A substitution, were associated with a loss of V2R–β-arrestin interactions, in agreement with the results obtained with the D2-like receptors and β2R. The V2R mutants also exhibited a loss in potency for agonist-stimulated G, protein–mediated signaling (cAMP accumulation), although maximum signaling activity was maintained. Human polymorphisms or genetic mutations that negatively affect V2R signaling result in nephrogenic diabetes insipidus (41–43); however, previous studies have emphasized diminished V2R-mediated cAMP accumulation or protein misfolding (41–43, 56, 57). Our current results now describe impaired β-arrestin recruitment associated with the V2R-Y205C/H5.38 polymorphisms, although further research is needed to determine how the loss of this pathway might be involved in nephrogenic diabetes.

Structural studies using crystallography and cryo-EM have provided new insights into the basis of GPCR activation and coupling with G proteins and β-arrestin signaling molecules. Different conformers of IL2 within the receptor appear to play a critical role in both of these interactions. Xu and colleagues have shown that in the rhodopsin–arrestin structure, the N and C domains of arrestin form a cleft between its middle and C-loops that the IL2 (in a helical conformation) of rhodopsin fits into (45). Mutation of select middle or C-loop residues in arrestin, or IL2 residues in rhodopsin, weakens rhodopsin–arrestin interactions (45). Conversely, in the rhodopsin–G,-cryo-EM structure (44), the IL2 of rhodopsin is in an extended loop (see above) and exhibits less extensive interactions with G,. In contrast, in the case of the β2R–G,-structure, the IL2 adopts a small two-turn helix that is important for G,-activation (58, 59). Dror and colleagues (60, 61) have provided evidence that arrestin activation is primarily achieved through interaction with the receptor core and intracellular loops of rhodopsin, specifically IL2 and, to a lesser degree, IL3.

Thus, the results of our MD simulations using the β2R as a model system, which predict an altered orientation of IL2 in response to the Y1995.38A mutation, are consistent with the observed loss or decrease in agonist-stimulated receptor–β-arrestin interactions. Further, our results with some agonists that exhibit limited β-arrestin recruitment to the D2R-F1895.38A, yet are incapable of promoting receptor internalization, suggest that this mutation cripples the receptor’s ability to activate β-arrestin. In contrast, altered IL2 conformations resulting from these mutations have a limited impact on G protein–mediated signaling, although agonist potentials for eliciting G protein activation were variably diminished. On the basis of the similar phenotypes of the aligned mutations in the highly homologous class A GPCRs investigated in this study, we propose that such a mechanistic pathway connecting an extracellular microswitch in the ligand binding site to IL2, which directly couples to signaling proteins, is commonly involved in biased signaling. Different ligands have been previously shown to produce distinct conformations of IL2 in other GPCRs (20, 62).

Choi et al. have described a β2R mutation near the juncture of TM5 and IL3 that biases the receptor for G protein–mediated signaling due to defective GRK5-mediated receptor phosphorylation, leading to diminished β-arrestin interactions (63). These investigators argued that the mutation did not affect intrinsic receptor–β-arrestin interactions because the fusion of a phosphorylated V2R peptide to the mutant β2R rescued its ability to undergo β-arrestin–mediated desensitization. Instead, they concluded that bias for or against β-arrestin–mediated signaling is mainly regulated through GRK-mediated receptor phosphorylation, which conceivably can be modulated by biased agonists. Although receptor phosphorylation by GRKs typically enhances β-arrestin association and its activation, this is not universal because GPCRs lacking C termini or phosphorylation sites can still recruit and activate β-arrestin (61, 64). We have previously shown that abrogation of GRK-mediated phosphorylation of the D1R (65) or D2R (38, 39) did not affect their ability to recruit and interact with β-arrestin. Thus, β-arrestin signaling bias can undoubtedly arise through different mechanisms that regulate β-arrestin interactions with the GPCR. For instance, Masureel et al. (66) have shown that a hydrogen-bond network between Ser204 and Asn293 in the β2R may underlie β-arrestin signaling bias for select β2R agonists.

In summary, our current study illustrates how structural perturbations in the extracellular ligand binding site can allosterically propagate to the intracellular surface of a GPCR and affect its ability to interact with signaling transducers, thus producing signaling bias. The further elucidation of structural determinants that underlie agonist-specific signaling states may assist in the rational design of novel functionally selective agents that can serve as improved therapeutic agents.

**MATERIALS AND METHODS**

**Materials and reagents**

MLS1547 was originally obtained from the National Institutes of Health Molecular Libraries Screening Center Network Library (27) and subsequently synthesized and verified for purity at the University of Kansas Specialized Chemistry Center by K. Frankowski (28). Gto1-Rluc8, Gβ1, Gγ2-mVenus, Gα12-Rluc8, CAMYEL biosensor, β-arrestin2-mVenus, human D2R-Rluc8, and human D3R-Rluc8 were gifts from J. Yano at Columbia University. Additional human receptor cDNAs (D4R, β2R, and V2R) were obtained at the cDNA Resource Center (www.cdna.org). Further receptor constructs and mutants were prepared by Bioinnovative (Rockville, MD). Constructs were prepared in pcDNA3.1 vectors, and inserts were verified by sequencing. LYN–recombinant green fluorescent protein (rGFP) (40) was a gift from M. Bouvier at the University of Montreal. All tissue culture media and supplies were obtained from Thermo Fisher Scientific (Carlsbad, CA). All other compounds and chemicals, unless otherwise noted, were obtained from Sigma-Aldrich (St. Louis, MO).

**Cell culture and transfection**

Human embryonic kidney (HEK) 293 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). Chinese hamster ovary (CHO)–K1-EA cells were cultured in Ham’s F12 media supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and hygromycin (300 μg/ml). Cells were grown at 37°C in 5% CO2 with 90% humidity. HEK293 cells were seeded in 100- or 35-mm plates and transfected overnight using a 1:3 ratio [1 μg of DNA:3 μl of polyethyleneimine (PEI)] diluted to 1 ml in nonsupplemented DMEM and added (100 μl/ml)
to the cells already in culture media. Media were replaced with complete media the following day. CHO-K1-EA cells seeded in 100- or 150-mm plates were transfected with TransIT-LT1 (Mirus Bio, Madison, WI) according to the manufacturer’s instructions. Media were replaced after 18 hours, and cells were plated for experiments conducted the following day. Concentrations of DNA are indicated for each experiment type.

**BRET assays**

HEK293 cells were transiently transfected with a BRET donor and corresponding BRET acceptor. Briefly, 4 × 10⁶ cells per plate were seeded in 100-mm dishes and incubated overnight. BRET experiments were performed 48 hours after transfection. The amounts of cDNA used for each type of BRET assay varied. β-Arrestin recruitment BRET assays used 1 μg of receptor-Rluc8 together with 5 μg of β-arrestin2–mVen corresponding BRET acceptor. Briefly, 4 × 10⁶ cells per plate were seeded on 100-mm dishes and incubated overnight. BRET signals were determined by calculating the ratio of the light emitted by mVen (535/30 nm) over that emitted by Rluc8 (475/30 nm) (BRET₁) using a PHERAstar plate reader (BMG LABTECH, Cary, NC). The net BRET values were obtained by subtracting the background ratio from untreated cells. Agonist-promoted BRET changes were expressed as a percentage of the maximum response of the WT receptor for each ligand.

**Lance assay for cAMP**

cAMP accumulation was measured by using the TR-FRET–based LANCE assay (PerkinElmer). Briefly, 4 × 10⁶ HEK293 cells per plate were seeded on 100-mm dishes and incubated overnight. Cells were then transfected with 5 μg of untagged receptor and 5 μg of CAMYEL biosensor using the PEI method described above. BRET experiments were performed 48 hours after transfection. On experiment day, cells were harvested, washed, and resuspended in DPBS containing 200 μM sodium metabisulfite and 5.5 mM glucose. Cells were plated in 96-well white, solid-bottom plates (Greiner Bio-One) and incubated in the dark for 45 min. For Gα₉-mediated adenyl cyclase inhibition, cells were pretreated for 5 min with 10 μM forskolin and 10 μM propranolol (to block endogenous β2R). Cells were then stimulated for 5 min with agonist, and BRET signal was determined by calculating the ratio of the light emitted by mVen (535/30 nm) over that emitted by Rluc8 (475/30 nm) (BRET²) using a PHERAstar plate reader (BMG LABTECH, Cary, NC). The net BRET values were obtained by subtracting the background ratio from untreated cells. Agonist-promoted BRET changes were expressed as a percentage of the maximum response of the WT receptor for each ligand.

Radioligand competition and saturation binding assays were conducted with slight modifications as previously described by our laboratory (27, 28). For competition binding experiments, $1.5 \times 10^6$ CHO-K1-EA cells were seeded in 100-mm dishes and incubated overnight. The next day, cells were transfected with 10 µg of indicated nontagged receptor construct using the TransIT-LT1 transfection reagent (Mirus Bio). For saturation binding experiments, $4 \times 10^6$ HEK293 cells were seeded in 100-mm dishes and incubated overnight. The next day, cells were transfected with 5 µg of indicated nontagged receptor along with 1 µg of Gαo–RLuc8, 5 µg of Gβγ2–mVen, and 4 µg of Gβδ2 or with 1 µg of indicated receptor tagged with RLuc8 along with 5 µg of β-arrestin2-mVen and 5 µg of GRK2 using TransIT-LT1 (Mirus Bio). Forty-eight hours after transfection, cells were dissociated from plates using Earle’s balanced salt solution (EBSS), and intact cells were collected by centrifugation at 900g for 10 min. Cells were resuspended and lysed with 5 mM tris-HCl and 5 mM MgCl₂ at pH 7.4 and resuspended in EBSS + CaCl₂ at pH 7.4. Cell lysates (100 µl, containing ~10 to 20 µg of protein, quantified by the Bradford assay) were incubated for 90 min at room temperature with the indicated concentrations of dopamine or MLS1547 and 0.2 nM $[^3]$H]methylyspiperone (for competition binding assays) or the indicated concentrations of $[^3]$H]methylyspiperone (for saturation binding assays) in a final reaction volume of 250 µl. Nonspecific binding was determined in the presence of 4 µM (+)-butaclamol. Bound ligand was separated from free by filtration through a PerkinElmer UniFilter-96 GF/C 96-well microplate using the PerkinElmer UNIFILTER-96 Harvester (PerkinElmer, Waltham, MA), washing three times with ice-cold assay buffer (1 ml per well). After drying, 50 µl of liquid scintillation cocktail (MicroScount PS; PerkinElmer) was added to each well, and plates were sealed and analyzed on a PerkinElmer Topcount NXT.


Cell surface receptor expression was determined using the membrane impermeable radioligand $[^3]$H]sulpiride in intact cell binding assays (38, 39). HEK293 cells ($13 \times 10^5$) were seeded in 150-mm dishes and incubated overnight. The next day, cells were transfected with 20 µg of nontagged D2R-WT or D2R-F189A plus 20 µg of GRK2 using the PEI method described above. Cells were seeded into poly-d-lysine–coated six-well plates 1 day before the assay at a density of $1 \times 10^6$ cells per well. Twenty-four hours after plating, cells were incubated in the presence of either 0.2 mM sodium metabisulfite (control) or 0.2 mM sodium metabisulfite plus 30 µM dopamine in DMEM for 1.5 hours at 37°C. Stimulation was terminated by rapidly cooling the plates on ice and washing the cells three times with ice-cold EBSS. Cells were then incubated with 0.5 ml of $[^3]$H]sulpiride in EBSS (final concentration, 7.3 nM) at 4°C for 3.5 hours. Nonspecific binding was determined in the presence of 7.5 µM (+)-butaclamol. Cells were washed three times with ice-cold EBSS and removed from plates with 0.5 ml of 1% Triton X-100 and 5 mM EDTA in EBSS. Samples were mixed with 2 ml of liquid scintillation mixture and counted with a Beckman LS6500 scintillation counter. Cells used to measure protein concentration were incubated with EBSS without $[^3]$H]sulpiride, and a Bradford assay was used to determine total cellular protein concentration per well. Data are represented as specific binding in fmol/mg protein.

Bias factor calculation

Dose-response data for apomorphine and rotigotine were fitted to the following form of the operational model of agonism (68) to allow the quantification of biased agonism as described in (69):

$$ Y = \frac{E_m - \text{basal}}{\left( \frac{x}{K_a} \right)^n} + \frac{\left( \frac{x}{K_a} \right)^n}{1 + \left( \frac{[A]}{K_a} \right)^n} $$

Where $E_m$ is the maximal possible response of the system, basal is the basal level of response, $K_a$ represents the equilibrium dissociation constant of the agonist (A), and $\tau$ is an index of the signaling efficacy of the agonist that is defined as $R_i/K_E$, where $R_i$ is the total number of receptors and $K_E$ is the coupling efficiency of each agonist-occupied receptor, and $n$ is the slope of the transducer function that links occupancy to response. The analysis assumes that the transduction machinery used for a given cellular pathway is the same for all agonists, such that the $E_m$ and transducer slope ($n$) are shared between agonists. D2R-WT and D2R-F189A dose-response data for apomorphine and rotigotine were fit for each pathway (G protein and β-arrestin) to determine values of $K_a$, $\tau$, and transduction coefficient $\log(\tau/K_a)$. ΔTransduction coefficients ($\Delta \log(\tau/K_a)$) were calculated for the G protein and β-arrestin data for each compound. $\Delta \log(\tau/K_a)$ is obtained by subtracting the Δtransduction coefficient for G protein from the Δtransduction coefficient for β-arrestin. The bias factors are the antilogos of $\Delta \log(\tau/K_a)$ and are shown in table S4.

MD simulation system setup and protocol

Initial coordinates of active-state β2R bound to agonist BI-167107 were downloaded from PDB entry 4LDE (30). The BI-167107–bound β2R crystal structure was determined using a β2R-T4 lysosome (β2R-T4L) fusion protein in which the T4L was fused to the N terminus of the receptor in the presence of camelid antibody fragment. We omitted T4L and camelid antibody fragment from all of our MD simulations. In addition, unresolved parts of IL3, N terminus, and C terminus were omitted from the simulations. Four mutations (M96T, M98T, N187E, and C265A according to UNIPROT numbering) that were introduced in the β2R crystal structure were mutated back to WT residues. Missing atoms of residues Lys60, Glu62, Lys149, Phe223, Gln224, Gln231, Lys263, Phe264, and Lys270 were added using Maestro (Schrödinger, LLC). Asp79, Glu122, and Asp130 were protonated as described previously (70).

Prepared receptor-ligand complexes were inserted into explicit palmitoyl-2-oleoylphosphatidylcholine (POPC) lipid bilayer environment using the Desmond MD System (version 4.5; D.E. Shaw Research, New York, NY). The system charges were neutralized, and 150 mM NaCl was added. Overall, the simulation systems consisted of ~107,889 atoms containing 297 lipid molecules, 58 sodium ions, 67 chloride ions, and 21,092 explicit water molecules. To elucidate how Ala substitution for Tyr5.38, which lies toward the extracellular side of β2R, affects β-arrestin interactions with the intracellular side of the receptor, in silico β2R-Y199A– WT MD simulation systems were prepared from representative frames from equilibrated β2R-WT MD simulation trajectories.

MD simulation systems were simulated using Desmond MD System (version 4.5; D.E. Shaw Research, New York, NY) with the OPLS3 force field (71) and TIP3P water model. The protein–membrane relaxation was carried out with a protocol modified from that developed...
REFERENCES AND NOTES


SUPPLEMENTARY MATERIALS
stke.sciencemag.org/cgi/content/full/13/617/eaaw5885/DC1
Fig. S1. D2R-WT and D2R mutants express to a similar extent using transient transfection.
Fig. S2. Other D2R agonists exhibit G protein bias at the D2R-F1895.38A mutant.
Fig. S3. - Arrestin and Go BRET signaling for D2R-WT and D2R-F1895.38A in response to full mutant.
Table S3. Mutants.
Table S4. - Arrestin–BRET and Go BRET signaling by D2R-WT and D2R mutants.
Table S5. Other D2R agonists exhibit G protein bias at the D2R-F1895.38A.
Table S6. Rotigotine- and apomorphine-stimulated internalization of the D2-WT and D2R-F1895.38A.
Table S7. D2R–BI-167107 interacting residues.
Table S8. J2R–Bl-167107 interacting residues.

View/request a protocol for this paper from Bio-protocol.


Acknowledgments: We thank M. Donegan for the excellent technical assistance and J. R. Lane for the helpful discussions. Funding: This study was supported by the Intramural Research Programs of the National Institute of Neurological Disorders and Stroke and the National Institute on Drug Abuse at the National Institutes of Health. Author contributions: M.S.-S., R.K.V., B.K.A.W., E.C.G., A.M.M., A.E.M., H.Y., R.B.F., L.S., and D.R.S. participated in the research design. M.S.-S., R.K.V., B.K.A.W., E.C.G., A.M.M., and A.E.M. conducted experiments. M.S.-S., R.K.V., B.K.A.W., E.C.G., A.M.M., A.E.M., and H.Y. performed data analyses. C.A.B. contributed research materials. M.S.-S., R.K.V., A.M.M., H.Y., R.B.F., L.S., and D.R.S. wrote or contributed to the writing of the manuscript. All authors contributed to and have approved the final manuscript. Competing interests: The authors declare that they have no competing interests. Data availability: All data needed to evaluate the conclusions in the paper are present in the paper or the Supplementary Materials.
A structural basis for how ligand binding site changes can allosterically regulate GPCR signaling and engender functional selectivity

Marta Sanchez-Soto, Ravi Kumar Verma, Blair K. A. Willette, Elizabeth C. Gonye, Annah M. Moore, Amy E. Moritz, Comfort A. Boateng, Hideaki Yano, R. Benjamin Free, Lei Shi and David R. Sibley

Sci. Signal. 13 (617), eaaw5885.
DOI: 10.1126/scisignal.aaw5885

A biasing position for GPCRs

GPCRs are the largest class of druggable receptors in the human proteome. Drugs that preferentially activate G protein– or β-arrestin–dependent signaling downstream of GPCRs are less likely to come with unwanted side effects. Using biochemical analyses, Sanchez-Soto et al. identified a specific conserved residue in the ligand binding site for multiple GPCRs that modulate β-arrestin–dependent signaling while minimally affecting that mediated by G proteins. Molecular dynamics simulations showed that mutations in this residue resulted in conformational changes that were expected to allosterically affect the interaction of the receptor with β-arrestin. These findings describe a mechanism by which changes in the ligand binding site of GPCRs can result in biased downstream signaling.

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