The dopamine D2 receptor can directly recruit and activate GRK2 without G protein activation

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Running Title: Direct GRK2 recruitment by the D2R

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Keywords: Pharmacology, G protein-coupled receptor (GPCR), Dopamine Receptor, GPCR Kinases, Bioluminescent Resonance Energy Transfer (BRET), Drug Action

ABSTRACT

The dopamine D2 receptor (D2R) is a G protein-coupled receptor (GPCR) that is critical for many central nervous system functions. The D2R carries out these functions by signaling through two transducers: G proteins and β-arrestins (βarrs). Selectively engaging either the G protein or βarr pathway may be a way to improve drugs targeting GPCRs. The current model of GPCR signal transduction posits a chain of events where G protein activation ultimately leads to βarr recruitment. GPCR kinases (GRKs), which are regulated by G proteins and whose kinase action facilitates βarr recruitment, bridge these pathways. Therefore βarr recruitment appears to be intimately tied to G protein activation via GRKs. Here, we sought to understand how GRK2 action at the D2R would be disrupted when G protein activation is eliminated and the effect of this on βarr recruitment. We used two recently developed biased D2R mutants that can preferentially interact either with G proteins or βarrs as well as a βarr-biased D2R ligand, UNC9994. With these functionally selective tools, we investigated the mechanism whereby the βarr-prefering D2R achieves βarr pathway activation in the complete absence of G protein activation. We describe how direct, G protein-independent recruitment of GRK2 drives interactions at the βarr-prefering D2R and also contributes to βarr recruitment at the WT D2R. Additionally, we found an additive interaction between the βarr-prefering D2R mutant and UNC9994. These results reveal that the D2R can directly recruit GRK2 without G protein activation and that this mechanism may have relevance to achieving βarr-biased signaling.

INTRODUCTION

G protein-coupled receptors (GPCRs) signal through two main transducers: G proteins and β-arrestins (βarrs) (1-3). G proteins regulate second messenger cascades such as cAMP and calcium that translate extracellular signals into cellular responses (4). Following prolonged or high concentrations of agonist stimulation, activated G proteins and their second messengers cause the recruitment and activation of GPCR kinases (GRKs) (5,6). GRKs phosphorylate serine and threonine residues on the intracellular domains of GPCRs, which promotes the recruitment of βarrs. The binding of βarr mediates receptor desensitization and endocytosis by sterically blocking further G-protein activation and by serving as an adaptor for the AP-2 and clathrin components of the endocytic machinery (7,8). Additionally, the scaffolding function of βarr enables it to bring together various other signaling components to mediate G protein-independent signaling (9-11).
Recently there has been a surge of interest in targeting GPCRs with biased or functionally selective ligands. These types of ligands favor the engagement of one signaling pathway over the other (12,13). Functionally selective drugs are predicted to have increased specificity of action by only engaging the therapeutically relevant pathway, while avoiding activation of potential side-effect inducing pathways (14-16). For the dopamine D2 receptor (D2R), selective targeting of the D2R/βarr signaling pathway may improve drugs used to treat schizophrenia, Parkinson’s disease, or other disorders (15,17,18).

However, the question of how fully activated D2R signal transduction cascades might be segregated remains unanswered. This is because maximal binding of βarr occurs at GPCR-phosphorylated and agonist-occupied GPCRs (19,20). The five non-visual GRKs that act on GPCRs rely on distinct plasma membrane recruitment mechanisms (21). For GRK2/3, which are the major GRKs that interact with D2Rs, plasma membrane recruitment is driven by an interaction between activated Gsα subunits and a C-terminal domain in GRK2/3 (5,22). Thus, the binding of βarr appears to be intimately linked to G protein-mediated recruitment of GRKs.

However, there is precedent for direct recruitment and activation of GRK2 by GPCRs. A notable study by Beautrait et al. (23), showed both G protein- and GPCR-mediated components of GRK2 recruitment and mapped the residues on the N-terminus of GRK2 involved in mediating recruitment and/or phosphorylation. Indeed, recent structural work at the β2AR and GRK5 demonstrates that GRK5 activation occurs via a rearrangement of GRK5’s RH/catalytic domain, which facilitates interaction between the GRK’s catalytic domain and the GPCR’s intracellular loops (24). These inter-domain interactions in GRKs are also necessary for holding them in inactive conformations (25). Therefore, interaction between the agonist-occupied GPCR and GRKs is necessary for activating GRKs and can facilitate their plasma membrane recruitment. Here, we sought to understand how GRK2 action at the D2R would be disrupted when G protein activation is eliminated and the effect of this on βarr recruitment.

We have previously generated two D2R mutants that display a high degree of signaling bias between the G protein and βarr pathways (26). The G protein preferring D2R ([Gprot]D2R) retains the ability to activate G-proteins while having markedly reduced βarr recruitment, whereas the βarr preferring D2R ([βarr]D2R) loses engagement of G-proteins while retaining βarr recruitment.

Additionally, our laboratory contributed to the development and characterization of a βarr-biased D2R ligand, UNC9994 (27). UNC9994 is based on the chemical scaffold of aripiprazole and has essentially no activity at the G protein pathway but retains partial agonism at the βarr2 pathway. The degree of UNC9994’s agonist/antagonist activity at the βarr pathway is dependent on GRK2 expression levels (28). When GRK2 expression is low, UNC9994 behaves more as a βarr-biased antagonist and when GRK2 expression is high it gains agonist activity at the βarr pathway (28).

In the present study, we combine these tools to investigate how loss of G protein activation impacts GRK2 engagement by the D2R and the resulting effect on βarr recruitment. We find that direct, G protein-independent recruitment of GRK2 by the D2R plays a significant role and is a means whereby the D2R can selectively promote D2R/βarr interactions. The implications of these findings were further explored using the GRK2-dependent ligand, UNC9994, where we found an additive interaction between it and the βarr-prefering D2R mutant. The elucidation of this and other mechanisms of achieving βarr bias should inform future efforts to design functionally selective ligands at the D2R and other GPCRs.

RESULTS

No Appreciable G-protein coupling of the [βarr]D2R

We first wanted to understand the mechanism whereby the [βarr]D2R achieves βarr recruitment without apparent G protein activation. Originally, the biased D2R mutants were characterized at the G protein pathway using the GloSensor assay, which measures downstream cAMP as a proxy for G protein activation (26). Therefore, we first tested whether potentially low levels of G-protein activation by the [βarr]D2R that
could be responsible for GRK2 recruitment to this receptor. We employed a recently described TGFr2 shedding assay to monitor G-protein activation over time (29). Importantly, this assay is sensitive enough to detect even basal GPCR activity (29). A cartoon diagram depicting how this assay works is presented in Figure 1A. We have adapted this assay to work with the fluorescent alkaline phosphatase substrate, 4-methylumbelliferyl phosphate. (For more details, see Experimental Procedures and (29).)

When performed with a Gα11/2 chimeric Gq protein, we observe the predicted shedding of the alkaline phosphatase into the culture media with the WT/D2R and GRK4/D2R (Fig. 1B). In contrast, there is no activity of the [βarr2]D2R at Gα11/2 (Fig. 1B), which matches the inactive D30A/D2R mutant (30). When performed with a Gα13 chimeric Gq protein (Fig. 1C), we observe similar results. A negative control chimeric Gq protein that lacks the 6 C-terminal residues also showed no activity with any D2R (Fig. 1D). When combined with our previous results using the GloSensor assay (26), we conclude that the [βarr2]D2R completely lacks functional G protein coupling. These data support the hypothesis that the [βarr2]D2R is capable of inducing βarr recruitment independently of G-protein activation.

Pertussis Toxin Reduces βarr2 Recruitment to WT/D2R and GRK4/D2R, but not to [βarr2]D2R

We next used bioluminescent resonance energy transfer (BRET) assays to measure the interaction between the D2R and βarr2 or GRK2 (31,32) (Fig. 2A). In order to remove the contribution of Gαi3-signaling (and its recruitment of GRK2/3) to the recruitment of βarr2, we pretreated the cells overnight with 200 ng/mL pertussis toxin (PTX). PTX ADP-ribosylates Gαi/o proteins, which completely inhibits Gαi/o activation and functional coupling (33-35). As shown with a βarr2 BRET assay in Figure 2, pretreatment with PTX reduced the efficacy and potency of βarr2 recruitment to the WT/D2R (Fig. 2B) and the efficacy of βarr2 recruitment to the GRK4/D2R (Fig. 2D). In contrast, the recruitment of βarr2 to the [βarr2]D2R was not affected by PTX (Fig. 2C). This result suggested that G protein-mediated translocation of GRK2 is not necessary for the recruitment of βarr2 to the [βarr2]D2R. The level of WT/D2R’s βarr recruitment has nearly the same efficacy as the [βarr2]D2R’s. Since the G protein inhibition caused by PTX did not eliminate βarr2 recruitment to the WT/D2R or the GRK4/D2R, an alternative mechanism may enable the D2R to recruit βarr2 in the absence of G protein signaling whereby GRK2 is directly recruited to the D2R.

Pharmacological and Genetic Inhibition of GRK2/3 Kinase Activity Diminishes βarr2 Recruitment to WT and Biased D2R Mutants

We next tested how inhibition of GRK2 kinase activity affected recruitment of βarr2 to the D2Rs. We and others have previously shown that recruitment of βarr2 to the WT/D2R is dependent on GRK2/3 kinase activity at both endogenous and heterologously-expressed D2Rs (28,36). We focused on GRK2 since it is expressed in the dopamine system (37) and D2R has been previously reported not to undergo significant GRK4/5/6-mediated phosphorylation in vitro (38). We first tested this dependence pharmacologically by pretreating the cells with a selective GRK2/3 inhibitor, Cmpd101. Cmpd101 works by binding to the kinase domain active site of GRK2/3 with an IC50 of ~35 nM (39) or 290 nM (40), depending on the assay used. Importantly, the IC50 for the related kinase GRK5 is >125 μM when tested with an in vitro kinase assay (39). Since we do not know the plasma membrane permeability of Cmpd101, we pretreated the cells for 60 minutes with 10 µM Cmpd101 for these assays to ensure the IC50 for GRK2/3 was reached. As shown in Figure 3, A-C, pretreatment with Cmpd101 markedly reduces βarr2 recruitment to the WT and biased D2R mutants. This result indicates that GRK2/3 kinase activity is necessary for the maximal recruitment of βarr2 to the D2R.

We then confirmed this phenomenon genetically by using a dominant negative K220R mutant GRK2 that lacks kinase activity, but can still bind Gβγ subunits and be recruited to the plasma membrane (41). Again, we observe that disruption of normal GRK2 kinase activity significantly reduces βarr2 recruitment to the D2R (Fig. 3, D-F). Together these results show that not only GRK2/3 recruitment but also kinase activity drives the recruitment of βarr2 to the WT and
mutant D2Rs. These results support the hypothesis that direct GRK2 recruitment to the D2R independently of G-protein activation drives its engagement of the βarr pathway.

In order to investigate the contribution of the other subfamily of GRKs (GRK4/5/6), we also performed the same assay with overexpression of GRK6 (Fig. 3, G-I). We did observe slight, statistically significant effects at each D2R mutant; however, the results are in contrast to the much larger effect we previously observed with GRK2 overexpression (28), where we observed a full log increase in βarr2 recruitment potency at the [WT]D2R that was reversed by Cmpd101. Therefore, we conclude that GRK2 is the key GRK necessary for βarr2 recruitment by the D2R.

Comparison of Agonist Induced D2R Phosphorylation with PTX or Cmpd101 Pretreatment

We next used 32P metabolic labeling to compare the effect of PTX or Cmpd101 pretreatment on the DA-induced phosphorylation of the D2Rs. As shown in Figure 4A, we observed no signal in our assay in the mock transfected condition indicating our assay was measuring 32P incorporated into the transfected D2Rs. In Figure 4B, we observed a statistically significant effect of DA stimulation alone versus the unstimulated condition at the [WT]D2R and [Gprot]D2R, but surprisingly not at the [βarr]D2R. However, this does not necessarily mean that the [βarr]D2R does not undergo DA-induced phosphorylation. As previously reported (38) the effect of DA alone was rather modest (50% above unstimulated) and we do not know which residues are phosphorylated. Nevertheless, these results do demonstrate that when pretreated with Cmpd101 or PTX, the effect of DA no longer differed from the unstimulated condition for any D2R (Fig. 4B), thereby indicating that both direct GRK2 kinase inhibition (Cmpd101) or pretreatment with PTX can reduce D2R phosphorylation.

Complete G Protein-Independent Recruitment of GRK2 to the [βarr]D2R

We next directly examined the role of G protein-dependent mechanisms in the recruitment of GRK2 to the D2Rs. By using a BRET assay for GRK2 recruitment (as schematically shown in Fig. 2A), we determined that PTX pre-treatment can significantly reduce the recruitment of GRK2 to the [WT]D2R (Fig. 5A). As expected based on our previous data, there was no effect of PTX on GRK2 recruitment at the [βarr]D2R (Fig. 5B). We also did not observe an effect of PTX at the [Gprot]D2R (Fig. 5C). These results are similar to the previous results assessing the effect of PTX on βarr2 recruitment.

As a control, we also assess the effect of Cmpd101 (10 μM) on GRK2 recruitment to the D2Rs (Fig. 5, D-F). As expected for a kinase inhibitor, Cmpd101 had no effect on GRK2 recruitment at any D2R.

We then tested whether a mutant GRK2 that lacks the C-terminal Gβγ binding domain could still be recruited to the D2Rs. We engineered a truncated form of the GRK2-eYFP construct that eliminates the last 28 amino acids from GRK2 before its in-frame fusion with eYFP, see Figure 5G. The V661 truncated GRK2 mutant has been previously shown to disrupt Gβγ-mediated translocation of GRK2 without affecting kinase activity (42).

We next assessed the interaction between the V661 truncated GRK2 BRET construct with the D2Rs. As predicted based on our previous results, we found that this GRK2 mutant was able to be dose-dependently recruited to the [WT]D2R and the [βarr]D2R (Fig. 5H). The largest degree of recruitment occurred at the [βarr]D2R, which supports the hypothesis that the [βarr]D2R can recruit GRK2 completely independently of G protein-dependent mechanisms. The [Gprot]D2R caused relatively little mutant GRK2 recruitment, which highlights the fact that GRK2 recruitment occurs in both a G protein dependent and independent manner. Taken together, these results clearly demonstrate that the D2R can directly recruit GRK2 independently of G-protein mechanisms.

Stimulation of the Mutant D2Rs with UNC9994 Demonstrates a Additive Effect Between [βarr]D2R and UNC9994

Since the agonist activity of UNC9994 at the βarr pathway appears to be dependent on GRK2 (28), we next tested its effect at the biased D2R mutants. We hypothesized that βarr2 recruitment induced by UNC9994 at the [βarr]D2R
would be greater than at the \([\text{WT}]\text{D2R}\). Indeed, we find that in HEK293T cells with endogenous GRK2 levels UNC9994 has a significantly increased ability to recruit \(\beta\text{arr}2\) at the \([\text{[arr]}]\text{D2R}\) compared to either \([\text{WT}]\text{D2R}\) or \([\text{Gprot}]\text{D2R}\) (Fig. 6A). This could be predicted based on both the GRK2 dependence of UNC9994 and the increased G protein-independent recruitment of GRK2 to the \([\text{[arr]}]\text{D2R}\) (28,32). When GRK2 was overexpressed (Fig. 6B), UNC9994 can now cause increased \(\beta\text{arr}2\) recruitment to the \([\text{WT}]\text{D2R}\) as previously observed (28). This effect was also apparent at the \([\text{[arr]}]\text{D2R}\) and to a smaller degree the \([\text{Gprot}]\text{D2R}\). As a test for the kinase specificity, we also overexpressed GRK6 (Fig. 6C) and found essentially no effect at the \([\text{WT}]\text{D2R}\) although we did observe statistically significant effects at the mutant D2Rs compared to WT, but not to the level of GRK2 overexpression.

We next compared the ability of UNC9994 to induce GRK2 recruitment to the wild-type and mutant D2Rs. We found that GRK2 recruitment was significantly enhanced at the \([\text{[arr]}]\text{D2R}\) (Fig. 6D). Again, this illustrates a case where a \(\beta\text{arr}\)-biased compound is engaging the \(\beta\text{arr}\) pathway through GRK2 in a G protein-independent manner. Taken together, these data show that UNC9994-induced \(\beta\text{arr}2\) recruitment is dependent on the inherent ability of the particular D2R to recruit GRK2.

**DISCUSSION**

The GPCR desensitization program enables cells to appropriately respond to high ligand concentrations by downregulating receptors. This linear pathway begins with G-protein activation and terminates after \(\beta\text{arr}\) binding. \(\beta\text{arrs}\) binding prevents G protein overactivation while also transducing G protein-independent signaling. GRK recruitment and activity links G-protein activation to \(\beta\text{arr}\) recruitment. Therefore, designing functionally selective ligands that solely activate either the G protein or \(\beta\text{arr}\) signaling arms is challenging because of the interdependence of these signaling events. Identifying receptor and pharmacological mechanisms that disentangle these pathways should lead to better drugs targeting GPCRs.

Here, we investigated how the D2R can achieve \(\beta\text{arr}\) recruitment via direct engagement and activation of GRK2 independently of activated G proteins. We found that the \([\text{WT}]\text{D2R}\) and biased D2R mutants (to varying degrees) can recruit GRK2 directly with a rather modest involvement of G\(\beta\gamma\)-mediated GRK2 translocation. In fact, the \([\text{[arr]}]\text{D2R}\) robustly recruits GRK2 without activating G proteins (Fig. 1, B & C) and therefore G protein inhibition has no effect on its recruitment of \(\beta\text{arr}2\) or GRK2 (Figs. 2C & 5B).

We have also confirmed a key role for GRK2 kinase activity at recruiting \(\beta\text{arr}2\) to the D2R, as GRK2 inhibition pharmacologically or genetically reduces \(\beta\text{arr}2\) recruitment at the D2R (Fig. 3). These results demonstrate that direct recruitment of GRK2 can play a key role during \(\beta\text{arr}\)-biased activation at the D2R. A caveat to these results is that they were obtained using overexpressed D2Rs which may overemphasize the importance of G protein-independent GRK2 recruitment at the D2R.

Therefore, we characterized the response of the biased D2R mutants to the \(\beta\text{arr}\)-biased D2R ligand UNC9994. We have previously observed that UNC9994’s degree of activity at D2R/\(\beta\text{arr}2\) is strikingly dependent on the expression levels of GRK2 both \textit{in vitro} and \textit{in vivo} (28). In the context of this work, the increased ability of the \([\text{[arr]}]\text{D2R}\) to directly recruit GRK2 enables UNC9994 to cause recruitment of \(\beta\text{arr}2\) to the \([\text{[arr]}]\text{D2R}\) even at endogenous GRK2 expression levels (Fig. 6A). This demonstrates that direct GRK2 recruitment is relevant to achieving \(\beta\text{arr}\)-biased signaling and suggests that this mechanism could be further exploited by forthcoming D2R ligands.

In conclusion, this study enhances our understanding of how a \(\beta\text{arr}\)-biased D2R mutant achieves its signaling profile through direct, G protein-independent recruitment and activation of GRK2. We also demonstrate that the D2R can recruit GRK2 with an unexpectedly large G protein-independent component. This work also provides proof-of-concept that direct recruitment of GRK2 to the D2R could be a means of tailoring the profile of a functionally selective D2R ligand. Often, the key contribution of GRKs in mediating the switch between G protein and \(\beta\text{arr}\) engagement is overlooked when screening for or assessing biased drugs. Structural work investigating the
mechanism of GRK5 activation by GPCRs and investigations of GRK2 activation (23,24) indicate that GRK activation is a key feature of GPCR signal transduction. Therefore, elucidating this and other mechanisms of selective G protein or βarr pathway engagement will inform future efforts to design functionally selective GPCR therapeutics.

**EXPERIMENTAL PROCEDURES**

**Plasmids**

For the BRET experiments, previous vectors encoding the *mus musculus* dopamine D2 receptor (long variant) in either the WT, Gprot, βarr, or D80A mutants was cloned in-frame the N1-Rluc vector (Perkin Elmer) (26). We modified the BRET constructs by replacing Rluc with RlucII on the D2Rs. The Rluc was mutated into RlucII by introducing two point mutations (C124A and M185V) via PCR mutagenesis. Subsequently, all DNA vectors were re-sequenced via Sanger sequencing (Eton Bioscience Inc., San Diego, CA) to verify the correct identity of the D2R mutants and to check for random mutations.

Additionally, the sensitivity of the D2R/βarr2 BRET experiments was further increased by cloning *mus musculus* βarr2 into the N1-mVenus vector (Addgene plasmid #54640) instead of the previously used N1-eYFP vector (Clontech, Mountain View, CA). This was done by PCR amplification of βarr2 with the following primers to add a 5’NheI site (5’ GGATCCCGGTTCTTCATCTTGGGCACCCG and 3’ CGGACCTGGAGGCGGTGC) and 3’BamHI site (5’ CGAGCTCAAGCTTCCAATTCGGCGCCATGG and a 3’ApaI site (5’ GGGCCCGGCAGAGGCCGAGCCGACCCG). This resulted in about a 2-fold increase in the maximal BRET signal versus the Rluc/eYFP construct pair (data not shown).

For the V661 Truncation GRK2-eYFP vector, *Bos taurus* WT GRK2 was PCR amplified with the following primers to add 5’ HindIII and 3’ BamHI sites immediately following V661 with the addition of GC to maintain the correct reading frame: 5’ CGAGCTCAAGCTTCCAATTCGGCGCCATGG and a 3’GGATCCCGGTTCTTCATCTTGGGCACCCG. This V661 truncated GRK2 PCR product was cloned into the same N1-eYFP vector that was used for the WT GRK2. The subsequent clones were verified by Sanger sequencing.

For the GRK2 K220R plasmid, the *Bos taurus* GRK2 K220R construct (Addgene plasmid #35403) was PCR amplified and ligated into pcDNA 3.1+ using 5’NheI and 3’NotI sites and the following primers: 5’ ATTTAACGTGGCTAGCGCCACCTGGCCGACCTGGAGGCGGTGC and 3’GGGCCCGCTCAGAGGCCGACTGCCC. The subsequent clones were verified by Sanger sequencing.

The *Bos taurus* WT GRK2 plasmid was used (43).

For the TGFα shedding assay, plasmids were the same as those described in (29), except for the D2Rs, which were the same as in (26).

For the D2R phosphorylation assay, plasmids were the same as in (26).

**Cells**

HEK293T cells obtained from the American Type Culture Collection (ATCC) were cultured and transfected via the standard calcium phosphate method and were used in all BRET assays. For the TGFα shedding assay, HEK293 cells that lack Gq/11, which were previously modified via CRISPR/Cas9 and characterized (44) in order to reduce non-specific shedding activity.

**Chemicals & Drugs**

Dopamine hydrochloride, ascorbic acid, 4-methylumbelliferyl phosphate (4-MUP) disodium salt, and DMSO were purchased from Sigma-Aldrich (St. Louis, MO). Cmpd101 was purchased from HelloBio (Princeton, NJ). Coelenterazine h and pertussis toxin were purchased from Cayman Chemical (Ann Arbor, MI). Hanks Balanced Salt Solution (HBSS) with Ca²⁺ and Mg²⁺ was purchased from Thermo Fisher Scientific (Waltham, MA). UNC9994 was synthesized as previously described (27). Phosphorus-32 radionuclide orthophosphoric acid in water (specific activity: 285.6 Ci/mg, concentration: 10 mCi/mL) was purchased from Perkin Elmer (Waltham, MA). Unless otherwise stated all other incidental chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Cmpd101 was initially dissolved in DMSO at 10 mM and subsequently diluted in HBSS. UNC9994 was initially dissolved...
in DMSO at 10 mM and subsequently diluted in Drug Dilution Buffer (HBSS supplemented with 20 mM HEPES, 0.3% BSA, 0.03% ascorbic acid) to maintain solubility of the compound. Dopamine was dissolved directly in HBSS supplemented with 0.03% ascorbic acid to prevent oxidation, except where compared directly to UNC9994, where the Drug Dilution Buffer was used instead.

**BRET Assays**

Performed as described previously using pre-determined ratios of RlucII and mVenus tagged proteins (31). Briefly, HEK293T cells were seeded at 70% confluence in a 6 well plate on the previous day and transfected via the calcium phosphate method using 0.4 µg of D2R-RlucII and either 2.5 µg βar2-mVenus or 1.0 µg GRK2-eYFP. The following day, cells were plated onto poly-D-lysine coated, clear bottom, white-walled 96-well plates using clear MEM supplemented with 10 mM HEPES, 1x GlutaMax, 2% FBS, and 1x Anti-Anti (BRET media). If cells were treated with PTX, they were incubated with 200 ng/mL overnight before the day of the experiment. If cells were treated with Cmpd101, cells were incubated with 10 µM for 1 hour prior to running the assay. On the day of the assay, the media was removed, a white vinyl sticker was placed on the bottom of the plate and HBSS was added to bring the final volume to 100 µL per well. A 10x concentration of colenterazine h (10 µL – final concentration ~4.7 µM) along with a 10x dose-response of ligand (10 µL) was added and the plates read on a Berthold Mithras LB 940 plate reader (Bad Wildbad, Germany). These readings were averaged per plate and the plate averages were combined in the graphs presented with the basal BRET ratio (unstimulated) subtracted off to give the Net BRET ratio. There was no significant difference between the D2R mutants at baseline in any of the assays.

**TGFα Shedding Assay**

Performed essentially as described in (29) with a difference in the alkaline phosphatase substrate and the use of G_{4α1} CRISPR-knockout cells (44). We used the fluorescent substrate, 4-Methylumbelliferyl phosphate (4-MUP), instead of p-Nitrophenyl Phosphate (p-NPP). This enabled us to measure the alkaline phosphatase using a ClarioStar plate reader (BMG Labtech, Ortenberg, Germany) equipped with a fluorescent reader, with excitation set at 360 nm (±10 nm) and emission at 450 nm (±15 nm). Data were collected over the course of 30 minutes. The shedding activity was calculated by dividing the amount of phosphatase activity present in the conditioned medium by the amount present on the cells plus the conditioned medium. These values were then vehicle subtracted and normalized (%) to the amount of alkaline phosphatase activity in the conditioned medium induced by 100 nM TPA for each transfection condition.

**D2R Phosphorylation Experiments**

These experiments were performed essentially as described previously (38,45) with slight modifications. 3xHA epitope-tagged D2R receptor constructs were transfected into HEK293T cells. The next day, cells were re-plated at high density onto poly-D-lysine coated 6 well plates in low-serum BRET media. If cells were treated with PTX, they were incubated overnight with 200 ng/mL. The following day, cells were metabolically labeled with 100 µCi of ^32P, in phosphate-free DMEM containing 20 mM HEPES and antibiotics/antimitotics at 37°C for 60 minutes. Cmpd101 (10 µM) or vehicle (diluted DMSO) was included during the metabolic labeling. Samples were then stimulated with 10 µM DA or vehicle for 10 minutes at 37°C. Then cells were rinsed in ice-cold PBS and then lysed with RIPA+ buffer (150 mM NaCl, 50 mM Tris, 5mM EDTA, 1% (v/v) NP-40, 0.5% (w/v) deoxycholate (Na salt), 0.1% (w/v) SDS, 50 mM NaF, 10 mM sodium pyrophosphate with supplemented with fresh EDTA-free protease inhibitor cocktail). Samples were then solubilized by rotating for 45 minutes at 4°C. Samples were then spun down to remove insoluble debris and transferred to a new tube containing 15 uL of anti-HA magnetic beads (Pierce™ Anti-HA Magnetic Beads, Cat#: 88836) and incubated with rotation for 1.5 hours at 4°C. Beads were then washed 2x with ice-cold RIPA+ buffer and then 1x with TBS-T. Then samples were eluted at 65°C for 10 minutes with Lamelli sample buffer containing 100mM DTT.

Samples were then loaded to a 4-12% Bis-Tris gel for SDS-page analysis (ThermoFisher
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Sci., Cat#: NP0323BOX). After mass separation on the gel samples were transferred to PVDF membranes (Thermo Sci., Cat#: 88585) and then the membranes were dried and subjected to autoradiography. Membranes were placed in cassettes containing intensifying screens (Kodak® BioMax® MS) and film (Kodak® BioMax® MR film). The cassettes were placed at -80°C for 48-72 hours before film development. The films were scanned using a film scanner and images were quantified using ImageJ (NIH). Following this, the same membranes were subjected to a western blotting procedure using a rabbit anti-HA tag primary antibody (Cell Signaling Tech, Cat#: 3724) and licor goat anti-rabbit 800 secondary (Li-Cor, Cat#: 926-32211). Membranes were then scanned on the Li-Cor near-infrared scanner and images were then quantified using ImageJ. Values were determined by normalizing the autoradiography signal to the anti-HA western blot for the same region and then normalizing to the unstimulated condition for each D2R mutant.

Curve Fitting & Statistical Analysis

Dose-response curves were fit using GraphPad Prism’s log (agonist) vs. response (three parameters) nonlinear fit function in order to calculate logEC50 and max efficacy values.

Statistical analysis was performed in Prism version 7.0 (GraphPad Software Inc., La Jolla, CA) as indicated in the text. Experiments testing the effect of a manipulation at each receptor were considered as one statistical unit for the purposes of ANOVA which was performed before Bonferroni-corrected t-tests. Each BRET or TGF shedding assay was performed in duplicate with at least 4 independent replicates.

Acknowledgements: We thank Lauren Rochelle for laboratory organizational support. Dr. Lawrence Barak, Dr. Joshua Snyder, and Jeffrey Smith for critically reading the manuscript and providing feedback. We thank Dr. Asuka Inoue for providing the plasmids used in the TGFα shedding assay and Jeffrey Smith for helpful advice regarding this assay. We thank Dr. Neil Freedman for sharing his 32P metabolic labeling procedure. We thank Dr. Jian Jin and his laboratory for kindly synthesizing and providing the UNC9994 for these studies. We also thank Dr. Michael Davidson and Dr. Robert Lefkowitz for the gifts of a plasmids encoding mVenus and GRK2 K220R via AddGene, respectively. Work for these studies was supported in part by NIDA Ruth L. Kirschstein NRSA F31 DA041160 to TFP, a Duke University Biological Sciences Undergraduate Research Fellowship to MIO, and the grants NIMH R37 MH073853 and U19 MH082441 to MGC.

Conflict of Interest: MGC has been a consultant for Omeros Corporation and owns stock in Acadia Pharmaceutical. MGC has received compensation in the form of honoraria for lecturing at various academic institutions. None of the above presents any conflicts of interest with the results described in the present paper.

Footnote: The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Author Contributions: TFP designed and performed experiments, cloned DNA vectors, analyzed data, and wrote the manuscript. MIO performed experiments and cloned DNA vectors. CR designed and performed experiments. SMP cloned DNA vectors, analyzed data, and wrote the manuscript. MGC designed experiments, analyzed data, and wrote the manuscript.
Direct GRK2 recruitment by the D2R

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FIGURE LEGENDS

Figure 1. Absence of G-Protein Coupling at the [barr]D2R. G-protein coupling for each D2R mutant was assessed via alkaline peroxidase activity released into the media following G$_{	ext{q}}$ activation of tumor necrosis factor-$	ext{c}$-converting enzyme (TACE). A) Cartoon of assay depicting coupling of D2Rs to chimeric G$_{	ext{q}}$ proteins. Chimeric G$_{	ext{q}}$ protein activation induces TACE activation, which cleaves and releases alkaline phosphatase coupled to TGF$_{	ext{a}}$ into the media where it can catalyze the dephosphorylation of 4-MUP thereby generating the fluorescent molecule, 4-MU. B) Results of the TGF$_{	ext{a}}$ shedding assay with Go$_{1,2}$-mimetic protein for each D2R mutant. Note: the [D80A]D2R could not be fitted to a curve and a value of zero was used for statistical comparisons. C) Results of TGF$_{	ext{a}}$ shedding assay with Go$_{3}$-mimetic protein for each D2R mutant. D) Results of TGF$_{	ext{a}}$ shedding assay with negative control Go$_{	ext{ACterm}}$ protein for each D2R mutant. Asterisks (****) denote statistical significance (P < 0.0001) for G-protein activation efficacy versus negative control [D80A]D2R for Bonferroni-corrected t-test following one-way ANOVA P < 0.0001. Error bars depict S.E. of 4 independent experiments.

Figure 2. Lack of Effect of PTX on barr2 Recruitment to [barr]D2R. A) Overview of BRET constructs showing agonist induced recruitment of barr2 or GRK2 to D2R, which brings YFP into close proximity to RlucII enabling BRET. Cells were pretreated overnight with vehicle (solid symbols) or 200 ng/mL PTX (open symbols) prior to stimulation with DA dose-response and BRET reading. B) Effect of PTX on barr2 recruitment to the [WT]D2R, C) [barr]D2R, D) [Gpro]D2R in response to DA. Pound signs (####) denote statistical significance (P < 0.0001) for changes in recruitment efficacy by Bonferroni-corrected t-test following two-way ANOVA P < 0.0001, while a dollar signs ($) denote statistical significance (P < 0.01) for change in recruitment potency by Bonferroni-corrected t-test following two-way ANOVA P < 0.0001. Error bars depict S.E. of 5 independent experiments.

Figure 3. Dependence on GRK2 Kinase Activity for barr2 Recruitment. (A-C): BRET assay measuring barr2 recruitment to D2Rs in HEK293T cells. Cells were pretreated with vehicle (solid symbols) or 10 µM Cmpd101 (open symbols) for 1 hour prior to stimulation with DA dose-response and BRET reading. A) Effect of 10 µM Cmpd101 on barr2 recruitment to the [WT]D2R, B) [barr]D2R, C) [Gpro]D2R in response to DA. (D-F): BRET assay measuring barr2 recruitment to D2Rs in HEK293T cells. Cells were co-transfected with either control pcDNA (solid symbols) or the dominant negative K220R GRK2 (open symbols) in addition to the D2R and barr2. Plates were stimulated with various doses of DA prior to BRET reading. D) Effect of K220R GRK2 on barr2 recruitment to the [WT]D2R, E) [barr]D2R, F) [Gpro]D2R in response to DA. (G-I): BRET assay measuring barr2 recruitment to D2Rs in HEK293T cells. Cells were co-transfected with either control pcDNA (solid symbols) or GRK6 (open symbols) in addition to the D2R and barr2. Plates were stimulated with DA dose-response prior to BRET reading. G) Effect of GRK6 overexpression on barr2 recruitment to the [WT]D2R, H) [barr]D2R, I) [Gpro]D2R in response to DA. Pound signs (###, ####) denote statistical significance (P < 0.001 or P < 0.0001) for change in recruitment efficacy by Bonferroni-corrected t-test following two-way ANOVA P < 0.0001, while a dollar signs ($) denote statistical significance (P < 0.01) for change in recruitment potency by Bonferroni-corrected t-test following two-way ANOVA P < 0.0001. Error bars depict S.E. of 4 independent experiments.

Figure 4. Effect of Cmpd101 and PTX on D2R Phosphorylation. Whole cell metabolic labeling with $^{32}$P was carried out in HEK293T cells transfected with HA-tagged D2Rs as indicated. Cells were preincubated with either 10 µM Cmpd101 for 1 hour (during the $^{32}$P labeling) or 200 ng/mL PTX overnight as indicated. Following 10-minute stimulation with 10 µM DA or vehicle, samples were subjected to the D2R autoradiography procedure as described in the Methods. A) Representative blot showing $^{32}$P autoradiography signal and anti-HA western blot, which served as a loading control. B) Quantification of
D2R autoradiography experiments. Autoradiography signal was normalized to the anti-HA western blot and then normalized to the unstimulated condition for each receptor. Asterisks (*, **) denote statistical significance at each receptor (P < 0.05, 0.01) for Bonferroni-corrected t-test comparing each treatment condition to the unstimulated condition following two-way ANOVA (P < 0.0001) for treatment. Error bars depict S.D. of 4 independent experiments.

Figure 5. Gbγ-Independent Recruitment of GRK2 to the [βarr]D2R. (A-C): BRET assay measuring GRK2 recruitment to D2Rs in HEK293T cells. Cells were pretreated overnight with vehicle (solid symbols) or 200 ng/mL PTX (open symbols) prior to stimulation with DA dose-response and BRET reading. A) Effect of PTX on GRK2 recruitment to the [WT]D2R in response to DA. B) Effect of PTX on GRK2 recruitment to the [βarr]D2R in response to DA C) Effect of PTX on GRK2 recruitment to the [Gprot]D2R in response to DA. Curves were fit using a nonlinear fit function. (D-F): BRET assay measuring GRK2 recruitment to D2Rs in HEK293T cells. Cells were pretreated for 1 hour with vehicle (solid symbols) or 10 µM Cmpd101 (open symbols) prior to stimulation with DA dose-response and BRET reading. (G & H): BRET recruitment of a truncated GRK2-eYFP to D2Rs in HEK293T cells. G) Cartoon of WT and V661 truncated GRK2 that were fused in-frame with eYFP to generate BRET constructs. The V661 truncated GRK2 lacks 28 amino acids at the C-terminal domain that is necessary for Gbγ-binding, but has retained kinase function. H) Recruitment of truncated GRK2 to the D2Rs in response to DA. For panels A-F, Pound signs (###, ####) denote statistical significance (P < 0.001 or P < 0.0001) for change in recruitment efficacy by Bonferroni-corrected t-test following two-way ANOVA (P < 0.0001), while a dollar signs ($) denote statistical significance (P < 0.01) for changes in recruitment potency by Bonferroni-corrected t-test following two-way ANOVA (P < 0.0001) or for panel H difference from [WT]D2R by Bonferroni-corrected t-test following one-way ANOVA (P < 0.01). Error bars depict S.E. of 4 independent experiments.

Figure 6. Additive Interaction Between UNC9994 and the [βarr]D2R. BRET assay measuring βarr2 or GRK2 recruitment to D2Rs in HEK293T cells. A) βarr2 recruitment to the D2Rs by UNC9994 at endogenous GRK levels. B) βarr2 recruitment to the D2Rs by UNC9994 with GRK2 overexpressed. C) βarr2 recruitment to the D2Rs by UNC9994 with GRK6 overexpressed. D) GRK2 recruitment to the D2Rs by UNC9994. Asterisks (*, ***, ****) denote statistical significance (P < 0.05, 0.001, 0.0001) by Bonferroni-corrected t-test following one-way ANOVA (P < 0.05) comparing each D2R mutant to the [WT]D2R’s maximum efficacy. Error bars depict S.E. of 4 independent experiments.
Figure 1

B) $G_{\alpha_1/2}$

C) $G_{\alpha_3}$

D) $G_{\alpha_{\Delta Cterm}}$

TGFα Shedding Assay at the D2Rs

A)
Figure 2

A) βarr2 Recruitment Assay at the D2Rs

B) $[\text{WT}]_{\text{D2R}}$

C) $[\beta\text{arr}]_{\text{D2R}}$

D) $[\text{Gprot}]_{\text{D2R}}$

βarr2 Recruitment Assay at the D2Rs

Vehicle
PTX

### ns

Log [DA] (M)

βarr2 Net BRET

$[\text{WT}]_{\text{D2R}}$

$[\beta\text{arr}]_{\text{D2R}}$

$[\text{Gprot}]_{\text{D2R}}$

Downloaded from http://www.jbc.org/
Figure 3

βarr2 Recruitment Assay at the D2Rs

A) [WT]D2R

B) [βarr]D2R

C) [Gprot]D2R

D) pcDNA K220R GRK2

E) pcDNA K220R GRK2

F) pcDNA K220R GRK2

G) pcDNA GRK6

H) pcDNA GRK6

I) pcDNA GRK6
### Figure 4

#### A)

| Transfection: | mock | [WT]D2R | [Barr]D2R | [Gprot]D2R |
|--------------|------|---------|---------|-----------|
| DA:          | -    | +       | +       | +         |
| Cmpd101:     | -    | -       | +       | -         |
| PTX:         | -    | -       | -       | +         |

50 kDa

Anti-HA WB

#### B)

![Graph showing Relative Value to Unstim](image)

- **Statistical Significance:** 
  - **:** p < 0.01
  - *: p < 0.05

50 kDa
Figure 5

GRK2 Recruitment Assay at the D2Rs

A) [WT]D2R

B) [βarr]D2R

C) [Gprot]D2R

D) [WT]D2R

E) [βarr]D2R

F) [Gprot]D2R

G) WT GRK2

H) V661 Trunc GRK2
Figure 6

βarr2 Recruitment Assay at the D2Rs

A) Endogenous GRKs

B) GRK2 Overexpressed

C) GRK6 Overexpressed

GRK2 Recruitment Assay at the D2Rs

D)
The dopamine D2 receptor can directly recruit and activate GRK2 without G protein activation

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J. Biol. Chem. published online February 27, 2018

Access the most updated version of this article at doi: 10.1074/jbc.RA117.001300

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