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Distinct GRK2 Recruitment Mechanisms Differentially Shape GPCR Signaling Bias

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Abstract

Biased GPCR agonists that selectively engage G proteins or arrestins show therapeutic promise, but their development has been challenging. Key to understanding biased signaling is GRK2, which phosphorylates many GPCRs and facilitates arrestin recruitment. Canonically, GRK2 binds GPCRs only after being recruited to the plasma membrane by G $\beta\gamma$, implying that arrestin recruitment requires prior G protein activation and challenging the feasibility of developing highly biased agonists. However, emerging evidence suggests that certain GPCRs, including dopamine D2 receptor, can bind GRK2 independently of G $\beta\gamma$. Here, we combined a robust GRK2 recruitment assay and functional analyses to show that G $\beta\gamma$ -dependent and -independent GRK2 recruitment have distinct dynamics, signaling consequences, and impacts on bias. We further demonstrate that G $\beta\gamma$ -independent GRK2 recruitment facilitates arrestin recruitment to diverse GPCRs, revising the canonical model of GRK2 function. These findings highlight the need to account for distinct GRK2 recruitment mechanisms in the design of biased GPCR agonists.

Introduction

G protein-coupled receptors (GPCRs) represent the largest superfamily of membrane proteins (>800 members in humans)¹. They are expressed in virtually every cell, respond to diverse stimuli, and are targets of ~36% of clinically approved medications¹. GPCRs facilitate G protein signaling by promoting the exchange of GDP for GTP with subsequent dissociation of G α and G $\beta\gamma$ subunits². They also recruit arrestins, which sterically block G protein coupling, promote internalization, and facilitate non-canonical signaling³. G proteins and arrestins can mediate distinct downstream physiological and behavioral effects⁴, offering the possibility that preferential targeting of one pathway could lead to better therapeutic outcomes. Despite intensive drug discovery efforts, robust G protein or arrestin biased agonists are not available for most GPCRs⁵.⁶ For example, biased agonism has been extensively studied at dopamine D2 receptor (D2R), which plays central role in health (learning, memory, movement)⁷ and disease (Parkinson's disease, schizophrenia, addiction)^{8, 9, 10}. However, existing biased agonists only partially activate the desired pathway while retaining activity at the undesired pathway^{11, 12, 13, 14, 15}. This, combined with the clinical failure of several high-profile biased agonists^{16, 17, 18}, underscores the urgent need to reevaluate current approaches to biased agonist drug discovery.

Biased agonism arises when ligands stabilize receptor conformations that favor specific transducers (G proteins, arrestins). Identifying conformational bias requires quantitative methods that can not only detect but also *directly compare* activity across distinct transducers. However, established methods for quantifying G protein^{19, 20, 21} or arrestin^{22, 23} activity (1) rely on non-steady state measurements that integrate signaling over time, and/or (2) amplify receptor responses, producing readouts that are distal to receptor-transducer coupling. As a result, apparent bias can be shaped by cellular context (system bias) and assay design (observational bias), obscuring true conformational bias. These effects can be partially corrected by analytical models²⁴ but remain a

major source of variability across laboratories¹⁵. Furthermore, many assays require modification to the intracellular domain of GPCRs^{22, 23}, which could interfere with transducer coupling. Thus, we argue that robust steady-state, non-amplified systems using unmodified receptors are essential for accurately quantifying conformational bias. Towards this goal, we recently developed a bioluminescence resonance energy transfer (BRET)-based arrestin recruitment assay that satisfies these criteria^{25, 26, 27}.

Beyond these methodological limitations, biased agonism is further constrained by the intrinsic complexity of GPCR signaling, which is governed by the actions of multiple regulatory proteins. Emerging evidence indicates that G protein-coupled receptor kinases (GRKs) play a key role in biased signaling, as their action is required for arrestin recruitment. GRKs are AGC kinases that phosphorylate serine and threonine residues in the receptor intracellular loops and C-terminus, creating a high-affinity docking site for arrestins^{28, 29}. They contain an N-terminal α -helix that binds the transmembrane bundle of the receptor^{30, 31}, an RGS homology (RH) domain, and a catalytic kinase domain. Based on sequence homology and function, GRKs are subdivided into the GRK1 subfamily (GRK1, GRK7), GRK2 subfamily (GRK2, GRK3), and GRK4 subfamily (GRK4, GRK5, GRK6)^{28, 29}. The primary functional distinction between these subfamilies is their subcellular distribution, which is determined by differences in the C-terminal domain of GRKs. Whereas GRK1/7 and GRK4/5/6 are constitutively tethered to membranes via ionic interactions and/or post-translational modifications, GRK2/3 are cytosolic proteins that are recruited via their pleckstrin homology (PH) domain to membrane-bound G $\beta\gamma$ subunits that are released from activated G proteins^{28, 29}.

Among the GRKs, GRK2 is particularly important due to its broad expression and abundance³². It is believed that GRK2 cannot bind and phosphorylate GPCRs until it is recruited to membranes by G $\beta\gamma$ ^{33, 34}. In support of this model, we recently showed that arrestin recruitment to selected GPCRs is inseparable from G protein activation due to the dependence of GRK2 recruitment on G $\beta\gamma$ release³⁵, calling into question whether it is feasible to develop biased agonists that exclusively promote G protein- or arrestin-dependent signaling for GRK2-coupled receptors.

Contrary to the prevailing paradigm, some GPCRs have been suggested to engage GRK2 independently of G $\beta\gamma$. While GPCR:GRK2 complexes have been purified in the absence of G $\beta\gamma$ ^{33, 34, 36, 37}, evidence for complexes in living cells is largely indirect^{38, 39}. Notably, recent work indicates that G_{i/o}-coupled receptors such as D2R can recruit GRK2 in cells that have been treated with pertussis toxin (PTX), which inactivates G_{i/o}^{40, 41}. This implies that these receptors can recruit arrestin in the absence of G protein activation, positioning them as targets for biased agonist drug discovery. In support of this idea, recent advances have led to GRK2-biased agonists for β 2 adrenergic receptor (β 2AR) that preferentially, albeit not exclusively, promote GRK2 over G protein and arrestin signaling⁴². Nevertheless, although prior work points to the existence of G $\beta\gamma$ -independent GRK2 recruitment, differences in the dynamics and function of G $\beta\gamma$ -dependent and -independent GRK2 recruitment have been largely unexplored due to a paucity of methods that enable a head-to-head comparison of their activity, limiting our understanding of their biological and therapeutic relevance.

Here, we developed a sensitive non-amplified BRET-based GRK2 recruitment assay that is directly comparable to parallel assays for G protein⁴³ and arrestin^{25, 26, 27}, together providing a robust toolkit for accurate quantification of conformational bias. Using D2R, we show that G $\beta\gamma$ -dependent and -independent GRK2 recruitment have distinct dynamics and are differentially regulated. Functional analyses revealed that G $\beta\gamma$ -dependent and -independent GRK2 recruitment differentially promote arrestin recruitment and produce distinct signaling outcomes. In addition, G $\beta\gamma$ -dependent and -independent GRK2 recruitment can have starkly differing effects on the

signaling bias of selected D2R ligands. Finally, we demonstrate that GRK2 can directly interact with diverse GPCRs in a constitutive and/or inducible manner, challenging the prevailing model that its recruitment is solely G $\beta\gamma$ -dependent and revealing new opportunities for tuning biased signaling. Together, these findings uncover the relevance of distinct modes of GRK2 recruitment, which is key to understanding GPCR function and the development of biased therapeutics.

Results

A highly sensitive BRET-based GRK2 recruitment assay

We first set out to develop a robust method to detect GRK2 recruitment. GRK2 recruitment to activated GPCRs is typically measured using direct BRET, wherein the receptor and GRK2 are tagged with complementary donor and acceptor tags²⁷. D2R activation enhances BRET between a fluorescent protein acceptor tagged-D2R (D2R-acceptor) and a NanoLuc donor tagged-GRK2 (GRK2-donor) (**Fig. 1a**). However, this assay requires modification to the C-terminus of the receptor, which could alter its function. This is of particular concern for D2R, which has a short C-terminus that is inserted into the plasma membrane via a palmitoylated cysteine and is thus sensitive to modification⁴⁴. To measure GRK2 recruitment to unmodified D2R, we developed a bystander BRET-based assay wherein the GRK2-donor is recruited to the plasma membrane in response to receptor activation, which would result in an increase in BRET between the GRK2-donor and a fluorescent protein acceptor that is anchored to the inner leaflet of the plasma membrane (mem-acceptor) (**Fig. 1b**). As intended, D2R activation in the GRK2 bystander BRET assay led to an increase in BRET (**Fig. 1c**). We next compared the direct BRET and bystander BRET assays. There was no difference in maximal dopamine-induced BRET (**Fig. 1d**) or the potency of dopamine between these assays (**Fig. 1e**), indicating that the bystander BRET assay performs as well as the direct BRET assay.

We previously developed an arrestin helper-interaction bystander BRET (hiBRET) assay that incorporates weakly interacting helper peptide Sp1 into a donor tagged-arrestin and the cognate helper peptide SH3 into the mem-acceptor (mem-acceptor-SH3), stabilizing their interaction and increasing the dynamic range of the assay^{25, 26, 27}. Analogously, we installed Sp1 into GRK2-donor (GRK2-donor-Sp1) and measured its ability to BRET with mem-acceptor-SH3 in response to the activation of D2R (**Fig. 1b**), comparable to a previous study⁴⁵. Compared to the direct BRET assay, the GRK2 bystander hiBRET assay displayed a ~13-fold greater maximal dopamine-induced BRET (**Fig. 1d**), only a <2-fold increase in the potency of dopamine (**Fig. 1e**), and no difference in the kinetics of GRK2 recruitment (**Fig. 1f,g**). Furthermore, GRK2 recruitment in response to D2R activation was faster than arrestin-3 (Arr3) recruitment (**Supplementary Fig. 1**), consistent with the dependence of arrestin recruitment on GRK2 action³⁵. Thus, the bystander hiBRET assay enables highly sensitive detection of GRK2 recruitment without substantially altering GRK2 function.

Discriminating between G $\beta\gamma$ -dependent and -independent GRK2 recruitment

Upon activation, D2R couples G proteins, generating of a pool of free G $\beta\gamma$ that are available to GRK2⁴⁶. However, D2R was also shown to bind GRK2 independently of G proteins⁴⁰. Together, this suggests that GRK2 can be recruited to both G $\beta\gamma$ and directly to D2R and, thus, the GRK2 bystander hiBRET assay may simultaneously measure both processes. We sought to differentiate between these modes of recruitment by selectively impairing either G $\beta\gamma$ -dependent or -independent GRK2 recruitment.

Pertussis toxin (PTX) was previously used to inactivate G $_{i/o}$ and impair G $\beta\gamma$ -dependent GRK2 recruitment in response to D2R activation⁴⁰. Prior to applying PTX in the GRK2 bystander hiBRET assay, we characterized its function in direct BRET assays between GRK2-donor-Sp1 and either D2R-acceptor or acceptor tagged-G $\beta_1\gamma_2$ (G $\beta_1\gamma_2$ -acceptor). Consistent with previous studies⁴⁰,

PTX reduced but did not abolish GRK2 recruitment to D2R (**Supplementary Fig. 2a,b**). Surprisingly, PTX did not abolish GRK2 recruitment to $G\beta_1\gamma_2$ (**Supplementary Fig. 2c,d**) even in the presence of increasing amounts of PTX cDNA (**Supplementary Fig. 2e**), which is likely due to residual low-level activation of PTX-insensitive G proteins. Thus, PTX reduces $G\beta\gamma$ -dependent GRK2 recruitment but cannot be used to selectively interrogate $G\beta\gamma$ -independent recruitment.

Instead, we turned to mutants of GRK2 that were previously shown to impair GRK2 recruitment to either $G\beta\gamma$ or GPCRs. We developed a GRK2-donor-Sp1 variant that contains a mutation in its PH domain (R587Q) that impairs $G\beta\gamma$ binding (**Fig. 2b**)⁴⁷. We also developed a GRK2-donor-Sp1 variant that lacks the N-terminal α -helix of GRK2 (Δ 2-15) (**Fig. 2a,b**), which is indispensable for GRK binding to activated GPCRs^{31, 48, 49}. As intended, GRK2(R587Q)-donor-Sp1 interacted with D2R-acceptor but not $G\beta_1\gamma_2$ -acceptor, whereas GRK2(Δ 2-15)-donor-Sp1 interacted with $G\beta_1\gamma_2$ -acceptor but not D2R-acceptor (**Supplementary Fig. 2f,g**).

$G\beta\gamma$ -dependent and -independent GRK2 recruitment have distinct dynamics

While direct D2R:GRK2 or $G\beta\gamma$:GRK2 BRET provide evidence that GRK2 can independently bind to either D2R or $G\beta\gamma$ (**Supplementary Fig. 2**), their limited sensitivity hinders precise measurement of the dynamics of GRK2 recruitment. These assays also require different acceptor proteins and thus cannot be compared directly. In contrast, the GRK2 bystander hiBRET assay measures GRK2(Δ 2-15) and GRK2(R587Q) recruitment to the common acceptor protein, membrane-acceptor-SH3.

In the hiBRET assay, both GRK2(Δ 2-15) and GRK2(R587Q) were recruited to the plasma membrane in response to dopamine, albeit to a lower extent than wildtype GRK2 (GRK2-WT) (**Fig. 2c,d**). The sum of GRK2(Δ 2-15) and GRK2(R587Q) recruitment approached but did not equal maximal GRK2-WT recruitment (~85%) (**Fig. 2c,d**), suggesting a degree of synergy between these two modes of recruitment. Surprisingly, GRK2(R587Q) recruitment was comparable in magnitude to GRK2(Δ 2-15) recruitment (**Fig. 2c,d**), inconsistent with the notion that GRK2 recruitment is exclusively driven by its binding to $G\beta\gamma$ ^{33, 34}. Furthermore, GRK2 recruitment was completely abolished when the Δ 2-15 and R587Q mutations were combined (**Fig. 2c,d**), emphasizing that only $G\beta\gamma$ and the receptor itself mediate GRK2 recruitment. Similar results were observed for GRK3 (**Supplementary Fig. 3**), indicating that D2R facilitates $G\beta\gamma$ -dependent and -independent recruitment of both members of the GRK2/3 subfamily.

GRK2-WT recruitment rapidly increased and then gradually decayed overtime (**Fig. 2c,e,f**). To better understand this kinetic profile, we compared the dynamics of GRK2(Δ 2-15) and GRK2(R587Q) recruitment. The onset of GRK2(R587Q) recruitment was ~6-fold and ~10-fold slower than that for GRK2-WT and GRK2(Δ 2-15) recruitment, respectively (**Fig. 2c,e**). In addition, GRK2(Δ 2-15) recruitment also decayed at approximately twice the rate of GRK2(R587Q) recruitment (**Fig. 2c,f**). Likewise, while dopamine initially more potently induced recruitment of GRK2(Δ 2-15) than GRK2(R587Q), this difference decreased over time due to a concurrent decrease in the potency of dopamine for GRK2(Δ 2-15) and an increase for GRK2(R587Q) (**Fig. 2g,h**). This contrasts with the potency of dopamine for GRK2-WT, which is stable and lies between that for GRK2(Δ 2-15) and GRK2(R587Q) (**Fig. 2g,h**). Thus, D2R-dependent GRK2 recruitment is the sum of two processes, $G\beta\gamma$ -dependent and -independent GRK2 recruitment.

$G\beta\gamma$ -dependent and -independent GRK2 recruitment are differentially regulated

$G\beta\gamma$ -dependent GRK2 recruitment is initially favored over $G\beta\gamma$ -independent, followed by a convergence in the magnitude of the recruitment via both mechanisms (**Fig. 3a**). We hypothesized that heterogeneities in G protein function underlie differences in the balance between $G\beta\gamma$ -dependent and -independent recruitment. First, we investigated whether the decay in $G\beta\gamma$ -

dependent GRK2 recruitment is due to the inactivation of G protein by regulators of G protein signaling (RGS), which would reduce the amount of free G $\beta\gamma$ available to GRK2. To test this, we inactivated endogenous G $\alpha_{i/o}$ with PTX and measured GRK2(Δ 2-15) recruitment in the presence of an overexpressed mutant of G α_{i1} that is PTX-resistant and insensitive to RGS (PTXR:G183S)⁵⁰. GRK2(Δ 2-15) recruitment decreased at a slower rate in the presence of PTXR:G183S than the RGS-sensitive G α_{i1} (PTXR) (**Fig. 3b,c**), indicating that the decay in G $\beta\gamma$ -mediated GRK2 recruitment is due to RGS-dependent inactivation of G protein. In contrast, there was no difference in GRK2(R587Q) recruitment to D2R in cells expressing RGS-sensitive or RGS-insensitive G α_{i1} (**Fig. 3b,c**). Thus, G $\beta\gamma$ -dependent GRK2 recruitment is faster than G $\beta\gamma$ -independent recruitment but is less stable due to the inactivation of G proteins.

D2R couples to G $_i$ and G $_{oA}$ ⁴⁶, which could differentially affect G $\beta\gamma$ -dependent and -independent GRK2 recruitment. To assess this, we inactivated endogenous G $_{i/o}$ using PTX and overexpressed PTX resistant-G α_{i1} or G α_{oA} . GRK2-WT was more efficiently recruited in the presence of G $_{oA}$ than G $_{i1}$ (**Supplementary Fig. 4a**), consistent with greater G $\beta\gamma$ release from G $_{oA}$ than G $_{i1}$ upon activation of D2R in a BRET-based G protein activation assay (**Supplementary Fig. 4b,c**). GRK2(Δ 2-15) recruitment was greater in the presence of G $_{oA}$ than G $_{i1}$, whereas GRK2(R587Q) recruitment was unaffected by either G protein (**Fig. 3d,e**). In addition, the potency of dopamine for GRK2(Δ 2-15) recruitment, but not for GRK2(R587Q) recruitment, was higher in the presence of G $_{oA}$ than G $_{i1}$ (**Supplementary Fig. 4d**). Furthermore, while the potency of dopamine for GRK2(Δ 2-15) in the presence of either G $_{i1}$ or G $_{oA}$ was initially higher than that for GRK2(R587Q), the reduction in potency for GRK2(Δ 2-15) resulted in a convergence in the potency of dopamine for GRK2(Δ 2-15) and GRK2(R587Q) (**Supplementary Fig. 4d**). These data indicate that the preference for G $\beta\gamma$ -dependent and -independent recruitment is highly dependent on G protein subtype.

G proteins are expressed to varying degrees across different intracellular organelles⁵¹, which could shift the balance between G $\beta\gamma$ -dependent and -independent recruitment. We tested this by replacing the plasma membrane-associated mem-acceptor-SH3 protein in the GRK2 bystander hiBRET assay with SH3-tagged BRET acceptors that are targeted to specific organelles, including the endosomes, golgi apparatus, endoplasmic reticulum, and mitochondria (**Fig. 3f**). We measured GRK2(Δ 2-15) or GRK2(R587Q) recruitment to each compartment in response to D2R activation in cells expressing organic transporter 2 (OCT2), which facilitates dopamine influx into intracellular organelles⁵². GRK2-WT recruitment to the plasma membrane accounted for more than half of total recruitment and was ~10-fold higher than recruitment to any individual intracellular compartment (**Fig. 3g**). GRK2(Δ 2-15) and GRK2(R587Q) recruitment was balanced in all intracellular organelles except endosomes, where GRK2(Δ 2-15) recruitment was preferred (**Fig. 3h**). Thus, D2R activation produces a similar balance of G $\beta\gamma$ -dependent and -independent modes of GRK2 recruitment in most but not all subcellular compartments.

G $\beta\gamma$ -dependent and -independent GRK2 recruitment have distinct functions

We next assessed the functional roles of G $\beta\gamma$ -dependent and -independent GRK2 recruitment. D2R was previously shown to fully recruit arrestin in the presence of PTX, suggesting that G $\beta\gamma$ -independent GRK2 recruitment is sufficient for arrestin recruitment⁴⁰. However, our results indicate that PTX does not fully abolish G $\beta\gamma$ -dependent GRK2 recruitment (**Supplementary Fig. 2**). Furthermore, these experiments were performed in a cell background wherein endogenous GRKs could compensate for deficits in function of G $\beta\gamma$ -independent GRK2 recruitment. To account for this, we measured D2R-mediated arrestin recruitment in HEK293 cells that are devoid of GRK2/3/5/6 and arrestin-2/3 (Δ Q:Arr2/3 cells)⁵³. GRK2(R587Q) enhanced Arr3 recruitment but with lower efficacy (**Fig. 4a,b**), slower kinetics (**Fig. 4a,c**), greater stability (**Fig. 4a,d**), and lower

sensitivity to dopamine than GRK2-WT (**Fig. 4e,f**), consistent with the dynamics of G $\beta\gamma$ -independent GRK2 recruitment (**Fig. 2**).

Not surprisingly, GRK2(Δ 2-15) had no effect on Arr3 recruitment (**Fig. 4a,b**) because it cannot bind and phosphorylate the receptor. To address this, we first compared the potency of dopamine for GRK2(Δ 2-15) and GRK2(R587Q) to its potency for Arr3 in the presence of GRK2-WT. Interestingly, the potency of dopamine for Arr3 closely matched that for GRK2(R587Q) but not GRK2(Δ 2-15) (**Supplementary Fig. 5a**), suggesting that the G $\beta\gamma$ -independent pathway better reflects arrestin recruitment. To test this more directly, we applied a low dose of dopamine (100 nM) to cells that co-expressed GRK2-WT and G α_A , which robustly promotes G $\beta\gamma$ -dependent GRK2 recruitment without engaging G $\beta\gamma$ -independent recruitment (**Supplementary Fig. 5b**). Arr3 was not recruited under these conditions (**Supplementary Fig. 5c,d**), demonstrating that GRK2 recruitment via G $\beta\gamma$ -mediated mechanism is not sufficient for arrestin recruitment and thus stronger activation with higher agonist concentrations is needed for the receptor to engage the kinase in a way that is conducive for Arr3 binding.

Arrestins facilitate receptor internalization via coated pits, acting as a bridge between GPCR and clathrin and AP2⁵⁴. To determine the effects of G $\beta\gamma$ -dependent and -independent GRK2 recruitment in this process, we used a novel hiBRET-based internalization assay that measures changes in D2R levels at the plasma membrane (**Supplementary Fig. 5e**). As expected, GRK2-WT overexpression enhanced dopamine-induced D2R internalization in Δ Q:Arr2/3-GRK cells, whereas GRK2(Δ 2-15) did not (**Fig. 4g**; **Supplementary Fig. 5f**). GRK2(R587Q) also had no effect on receptor internalization (**Fig. 4g**; **Supplementary Fig. 5g**) despite being able to facilitate arrestin recruitment. We hypothesized that this was due to the inability of GRK2(R587Q) to fully promote arrestin recruitment (**Fig. 4a,b**). We tested this by applying a sub-saturating dose of dopamine (316 nM) to GRK2-WT-expressing cells, which results in comparable Arr3 recruitment to that in GRK2(R587Q)-expressing cells that are exposed to saturating dopamine (100 μ M) (**Supplementary Fig. 5g**). D2R internalization was not observed under these conditions (**Fig. 4g**; **Supplementary Fig. 5f**), confirming that GRK2(R587Q) does not promote a sufficient level of arrestin recruitment to drive receptor internalization.

Next, we assessed the roles of G $\beta\gamma$ -dependent and -independent GRK2 recruitment on G protein function. We first measured the effect of the overexpression of GRK2-WT and its mutants in the BRET-based G protein activation assay (**Supplementary Fig. 4b**). GRK2-WT and GRK2(Δ 2-15) strongly inhibited G protein activation (**Fig. 4h**; **Supplementary Fig. 6a**), consistent with their ability to bind free G $\beta\gamma$. In contrast, GRK2(R587Q) inhibited G protein activation to a lesser degree (**Fig. 4h**; **Supplementary Fig. 6a**). Analogous effects were observed on downstream G protein signaling. Acute D2R-mediated G protein-inwardly rectifying potassium channel (GIRK) activation was almost completely abolished by GRK2-WT or GRK2(Δ 2-15) (**Fig. 4i**; **Supplementary Fig. 6b,c**), whereas GRK2(R587Q) only partially reduced maximal current, slowed the on-kinetics of channel activation, and had no effect on the stability of the response (**Fig. 4i**; **Supplementary Fig. 6c-e**). Furthermore, D2R-mediated cAMP inhibition⁵⁵, a slower G protein-dependent process, was strongly attenuated by GRK2-WT overexpression, consistent with previous studies⁵⁶ (**Fig. 4j**; **Supplementary Fig. 6f-h**)⁵⁶. While GRK2(Δ 2-15) was fully inhibitory (**Fig. 4j**; **Supplementary Fig. 6f-h**), GRK2(R587Q) only partially reduced D2R-mediated cAMP inhibition (**Fig. 4j**; **Supplementary Fig. 6f-h**). Collectively, these data indicate that GRK2 inhibits G protein signaling through two distinct interactions — one with G $\beta\gamma$ and another directly with the receptor — with the G $\beta\gamma$ interaction exerting the stronger negative effect.

Finally, we explored the effect of G $\beta\gamma$ -dependent and -independent GRK2 recruitment on extracellular signal-regulated kinase (ERK), which is regulated by both G protein and arrestin⁵⁷.

GRK2-WT and GRK2(Δ 2-15) decreased ERK activity according to a BRET-based ERK biosensor⁵⁸ (**Fig. 4k; Supplementary Fig. 7a**). In contrast, GRK2(R587Q) enhanced ERK activity (**Fig. 4k; Supplementary Fig. 7b**).

G β γ -dependent and -independent GRK2 recruitment drive distinct biased signaling profiles

We evaluated whether G β γ -dependent and -independent GRK2 recruitment are differentially promoted by various synthetic unbiased and biased D2R ligands (**Fig. 5a**). Like dopamine, D2R agonists recruited more GRK2-WT than GRK2(Δ 2-15) or GRK2(R587Q) (**Fig. 5b-d; Supplementary Fig. 8a,b**). However, while dopamine promoted comparable GRK2(Δ 2-15) and GRK2(R587Q) recruitment, several compounds preferred GRK2(Δ 2-15) over GRK2(R587Q) (**Fig. 5b-d; Supplementary Fig. 8b**). This trend was most pronounced for the anti-Parkinsonian medication bromocriptine⁵⁹, which fully promoted GRK2(Δ 2-15) recruitment but had almost no effect on GRK2(R587Q) (**Fig. 5b-d; Supplementary Fig. 8a**). Notably, no compound strongly preferred GRK2(R587Q) (**Fig. 5d; Supplementary Fig. 8b,c**), suggesting that G β γ -independent GRK2 recruitment remains underutilized in the development of arrestin biased D2R ligands. Furthermore, GRK2(Δ 2-15) recruitment was tightly coupled to G β γ release (**Fig. 5c; Supplementary Fig. 8d-f**), underscoring the challenge of dissociating G protein activation from GRK2 recruitment in efforts to develop G protein biased agonists.

Next, we determined the impact of G β γ -dependent and -independent GRK2 recruitment on the preference of D2R agonists for G protein or arrestin (**Supplementary Fig. 9a**). G protein signaling is often measured using downstream signaling assays (e.g., cAMP, Ca²⁺) that are highly sensitive to weak partial agonism due to multiple layers of signal amplification. However, the arrestin recruitment assay used in this study is a non-amplified system that reports on the interaction between one receptor and one arrestin (**Supplementary Fig. 1**). To more accurately compare G protein and arrestin signaling, we leveraged a BRET-based G α subunit recruitment assay⁴³ that is analogous in design to the arrestin recruitment assay (**Supplementary Fig. 9b**). D2R agonists such as aripiprazole were unbiased regardless of whether arrestin recruitment was facilitated by GRK2-WT or GRK2(R587Q) (**Fig. 5e; Supplementary Fig. 9c-g**). However, agonists such as bromocriptine favored G α when arrestin recruitment was driven by GRK2(R587Q) but showed the opposite preference when arrestin was driven by GRK2-WT (**Fig. 5e; Supplementary Fig. 9c-g**), indicating that G β γ -dependent and -independent GRK2 recruitment can have opposing effects on biased signaling.

Existing D2R biased agonists do not meaningfully engage G β γ -independent GRK2 recruitment

MLS1547 was previously reported to be a G protein biased ligand that fully activates G protein while exhibiting minimal arrestin activity¹³. Although MLS1547 favored GRK2(Δ 2-15) over GRK2(R587Q), its engagement of both recruitment mechanisms was weak (**Fig. 5b-d; Supplementary Fig. 9c-g**), raising the possibility that its apparent G protein bias stems from inefficient GRK2 recruitment. However, prior studies assessed MLS1547-induced G protein signaling in an amplified cAMP inhibition assay^{13, 60}, which could overestimate intrinsic efficacy. When tested in the BRET-based G α recruitment assay, MLS1547 had very low activity compared to dopamine (~6% of dopamine E_{max}) (**Fig. 5c; Supplementary Fig. 9c,d,g**). Similarly, this compound promoted weak but measurable Arr3 recruitment in the presence of GRK2-WT (~7% of dopamine E_{max}), albeit it had no effect with GRK2(R587Q) (~1% of dopamine E_{max}) (**Fig. 5c; Supplementary Fig. 9e-g**). Thus, MLS1547 is a weak partial agonist that promotes balanced G protein and arrestin recruitment, with arrestin binding being supported exclusively by G β γ -dependent GRK2 recruitment (**Fig. 5E; Supplementary Fig. 9e-g**).

The reported arrestin biased D2R agonist UNC9994¹¹ promoted low but comparable GRK2(Δ 2-15) and GRK2(R587Q) recruitment (**Fig. 5b-d**). Thus, compared to other D2R compounds, UNC9994 uniquely displays a balanced GRK2 recruitment profile analogous to dopamine. Nevertheless, UNC9994 promoted weak G α recruitment (~4% of dopamine E_{max}) and GRK2-WT-dependent arrestin recruitment (~6% of dopamine E_{max}), while having almost no effect on arrestin in the presence of GRK2(R587Q) (~2% of dopamine E_{max}) (**Fig. 5c; Supplementary Fig. 9e-g**). These results indicate that UNC9994-induced arrestin recruitment is predominantly driven by G $\beta\gamma$ -dependent GRK2 recruitment (**Supplementary Fig. 9b,d,e**). Taken together, despite being reported to have opposing biased signaling profiles, MLS1547 and UNC9994 have nearly identical functional effects. Moreover, neither drug can promote arrestin recruitment independently of G $\beta\gamma$.

Diverse GPCRs recruit GRK2 independently of G $\beta\gamma$

We next compared G $\beta\gamma$ -dependent and -independent GRK2 recruitment across the GPCR superfamily. To test this, we measured GRK2-WT, GRK2(Δ 2-15), or GRK2(R587Q) recruitment to 25 additional GPCRs spanning different receptor families (Family A-C) and G protein coupling preferences (G $_{i/o}$, G $_{s/olf}$, G $_{q/11}$) (**Fig. 6a**). Agonist-induced GRK2 recruitment varied considerably across receptors and was highest at D2R (**Fig. 6b; Supplementary Fig. 10a**). Furthermore, GPCRs such as serotonin 2A receptor (5HT $_{2A}$ R) showed little to no recruitment of either GRK2 mutant (**Fig. 6b-d; Supplementary Fig. 10a,b**). Other receptors recruited GRK2(Δ 2-15) but not GRK2(R587Q) (**Fig. 6b-d; Supplementary Fig. 10a,b**), consistent with canonical GRK2 recruitment to G $\beta\gamma$. However, ~38% of the GPCRs that were tested recruited both GRK2(Δ 2-15) and GRK2(R587Q) in response to agonist (**Fig. 6b-d; Supplementary Fig. 10a,b**), indicating that agonist-induced G $\beta\gamma$ -independent GRK2 recruitment is more widespread than previously believed. While some of these receptors preferred GRK2(Δ 2-15) over GRK2(R587Q) (**Fig. 6b-d; Supplementary Fig. 10a,b**), others displayed balanced GRK2(Δ 2-15) and GRK2(R587Q) recruitment (**Fig. 6b-d; Supplementary Fig. 10a,b**), analogous to D2R. Only one receptor, metabotropic glutamate receptor 5 (mGluR5), favored GRK2(R587Q) over GRK2(Δ 2-15) albeit modestly (**Fig. 6b-d; Supplementary Fig. 10a,b**). Furthermore, agonist-driven GRK2(R587Q) recruitment and GRK2(R587Q)-dependent Arr3 recruitment were positively correlated (**Fig. 6e**), evidence that agonist-induced G $\beta\gamma$ -independent GRK2 recruitment facilitates arrestin recruitment to multiple receptors.

GPCRs have been shown to constitutively interact with arrestins⁶¹, raising the possibility that basal arrestin recruitment depends on G $\beta\gamma$ -independent GRK2 pre-coupling to receptor. Indeed, the GPCRs in our panel displayed varying degrees of basal association with GRK2(R587Q) (**Supplementary Fig. 10c**). Moreover, basal GRK2(R587Q) recruitment was positively correlated with basal Arr3 recruitment in the presence of GRK2(R587Q) (**Fig. 6f**), emphasizing the functional importance of constitutive G $\beta\gamma$ -independent GRK2 recruitment. Interestingly, agonist-induced and basal GRK2(R587Q) recruitment were negatively correlated (**Fig. 6g**). For example, D2R had among the lowest basal interactions with GRK2(R587Q) (**Supplementary Fig. 10c**) while facilitating the most agonist-induced recruitment (**Fig. 6b; Supplementary Fig. 10a**), implying that GPCRs that have a low basal interaction with GRK2 are more likely to inducibly recruit the kinase.

Interestingly, some GPCRs promoted GRK2(Δ 2-15) recruitment but lowered their basal association with GRK2(R587Q) in response to their endogenous ligands (**Fig. 6b-d; Supplementary Fig. 10b**), indicating that receptor activation can drive GRK2 recruitment via G $\beta\gamma$ while concurrently diminishing the basal receptor:GRK2 interaction. This was the case for dopamine D4 receptor (D4R) (**Fig. 6b,c; Supplementary Fig. 10a,b**), which may underly the inability of this receptor to recruit arrestin in response to dopamine^{62, 63}. Taken together, these

results show that GPCRs can promote G $\beta\gamma$ -independent GRK2 recruitment but vary considerably in their ability to engage this pathway in a constitutive and inducible manner.

Among tested GPCRs, mGluR1 had one of the highest levels of basal association with GRK2(R587Q) (~3.4-fold higher than that for D2R) (**Supplementary Fig. 10c**), consistent with the observation that this receptor immunoprecipitates with GRK2 independently of agonist stimulation^{64, 65}. To further probe basal mGluR1:GRK2 association, we applied glutamate, which produced a slight enhancement in the interaction. In contrast, the negative allosteric modulator FITM partially reduced it (**Fig. 6H; Supplementary Fig. 10d**). These results indicate that basal mGluR1:GRK2(R587Q) association shows only limited pharmacological sensitivity. We then compared GRK2-WT, GRK2(Δ 2-15), GRK2(R587Q), and the double mutant GRK2(Δ 2-15:R587Q) in the hiBRET GRK2 assay. All GRK2 variants exhibited high basal association with mGluR1, albeit there was a modest reduction in this interaction with the double mutant (**Fig. 6i**). This indicates that mGluR1 robustly engages GRK2 through an additional, undefined mechanism distinct from the receptor binding-N-terminal α -helix and G $\beta\gamma$ -binding PH domain. More broadly, high basal GRK2(R587Q) interactions were also observed with structurally and functionally divergent receptors (**Supplementary Fig. 10c**), suggesting that GPCRs may form basal contacts with GRK2 through multiple mechanisms. These findings underscore the need to define the molecular basis of GPCR:GRK2 interactions, particularly for successful discovery of biased agonists.

Discussion

While biased GPCR agonists have immense potential, they have produced little therapeutic impact, with the only approved biased drug oliceridine being restricted to narrowly defined clinical settings⁶⁶. The problem is that many biased ligands only partially favor the desired signaling pathway, which may in part be due to the canonical dependence of arrestin recruitment on G protein activation and subsequent G $\beta\gamma$ -dependent GRK2 recruitment. A possible solution to this problem is a non-canonical G $\beta\gamma$ -independent GRK2 recruitment pathway, which could in principle be attenuated to enhance G protein bias and harnessed to promote arrestin bias. However, G $\beta\gamma$ -independent GRK2 recruitment is understudied, limiting our understanding of its therapeutic value. Here, we show that a Family A GPCR D2R itself recruits comparable levels of GRK2 to G $\beta\gamma$. This contradicts the prevailing paradigm that G $\beta\gamma$ is necessary for GRK2 recruitment. Furthermore, we found that G $\beta\gamma$ -dependent and -independent recruitment mechanisms are differentially utilized by diverse compounds and receptors.

Physiological relevance of distinct GRK2 recruitment mechanisms

G $\beta\gamma$ -dependent GRK2 recruitment has a faster onset and requires lower agonist concentrations than direct GRK2-receptor recruitment, likely because G $\beta\gamma$ release reflects signal amplification (*each receptor activates many G proteins*) whereas GRK2 recruitment is a stoichiometric process (*each receptor can only bind one GRK2*). Nevertheless, this raises the possibility that these mechanisms are differentially engaged under physiological conditions. For example, extracellular dopamine fluctuates between nanomolar concentrations at baseline (*tonic release*) and transient micromolar bursts (*phasic release*) in response to specific stimuli such as rewards⁶⁷. Tonic dopamine may be sufficient to facilitate D2R-mediated G $\beta\gamma$ -dependent recruitment to the plasma membrane. However, these concentrations are not likely to support concurrent GRK2 binding to receptor and subsequent arrestin recruitment. In contrast, G $\beta\gamma$ -independent recruitment may require repeated trains of phasic release. GRK2 recruitment could also shift towards the G $\beta\gamma$ -independent pathway in response to drugs of abuse (cocaine, amphetamine), which greatly increase extracellular dopamine⁶⁸. Many GPCRs experience prolonged exposure to their endogenous ligands, implying that G $\beta\gamma$ -independent GRK2 recruitment could be a widespread feature of GPCR regulation.

A key finding of this study is that Gβγ-dependent and -independent GRK2 recruitment are functionally distinct. Gβγ-dependent recruitment drives canonical arrestin recruitment, which sterically blocks G protein signaling and facilitates receptor internalization. However, we show that the binding of GRK2 to Gβγ directly suppresses G protein signaling, consistent with the actions of the GRK2 PH domain carboxyl-terminal peptide (βARKct)⁶⁹. In contrast, Gβγ-independent GRK2 recruitment promotes only partial arrestin engagement, moderate G protein inhibition, and no internalization. We further show that Gβγ-dependent and -independent GRK2 recruitment exert opposing effects on ERK signaling, which is reportedly controlled by diverse mechanisms including G proteins and arrestins⁵⁷. Prior work demonstrates that G protein signaling can activate ERK in the absence of arrestins⁷⁰, suggesting that Gβγ-dependent GRK2 recruitment suppresses ERK by limiting G protein availability. Conversely, arrestins are insufficient to activate ERK without concomitant G protein signaling⁷¹, implying that Gβγ-independent GRK2 recruitment modulates, rather than directly drives, ERK activity. Together, our findings reveal a functional dichotomy wherein Gβγ-dependent GRK2 recruitment strongly constrains both G protein and ERK signaling, whereas Gβγ-independent recruitment dampens G protein signaling while potentiating ERK activity.

Gβγ-independent GRK2 recruitment is multimodal

The use of the G_{i/o}-inactivating toxin PTX overestimated the magnitude of D2R-mediated Gβγ-independent recruitment because PTX cannot fully eliminate Gβγ-dependent recruitment⁴⁰. Here using cleaner tools – GRK2 mutants – we confirmed that D2R recruits GRK2 independently of Gβγ and showed that this receptor is very efficient at using this mechanism. Building upon this result, we revealed an additional layer of GRK2 function – GPCRs vary not only in agonist-induced GRK2 recruitment but also in their basal association with the kinase. D2R has a low basal association with GRK2 yet exhibits robust GRK2 recruitment in response to agonist. Conversely, mGluR1 has a high basal association with GRK2, which may underlie rapid and robust GRK2-dependent desensitization of this receptor³⁹. Surprisingly, the GRK2 N-terminus does not contribute to this interaction, indicating that GRK2 is pre-coupled to mGluR1 via an alternative yet undetermined binding site(s). Previous studies show that mGluR1 directly interacts with an isolated GRK2 RH domain⁷² independently of Gα_q binding to GRK2⁶⁵, suggesting that the RH domain may contribute to this interaction. The corresponding binding site in mGluR1 is still unknown. In summary, our findings indicate that Gβγ-independent recruitment can arise not just from the GRK2 N-terminus but also through additional interactions, underscoring the need to define their molecular determinants and modulatory potential.

Leveraging distinct GRK2 recruitment mechanisms in biased agonist drug discovery

The central challenge in biased agonist drug discovery is to develop drugs that strongly activate the desired pathway while avoiding the undesired ones. Here, we outline new design principles for developing next-generation biased agonists. First, accurate quantification of bias is essential. Amplified assay systems can obscure intrinsic efficacy and artificially inflate bias. Although analytical pharmacology can partially mitigate this issue²⁴, the implementation of non-amplified highly sensitive assays such as those used in this study provide a complementary and potentially more reliable readout of bias, particularly for quantifying conformational bias independently of system bias.

Second, GRK2 recruitment mechanisms should be considered when designing biased agonists. In principle, G protein biased agonists that minimize Gβγ-independent GRK2 recruitment should be less likely to promote arrestin recruitment, whereas arrestin biased agonists that promote this pathway should support arrestin recruitment without requiring prior G protein activation (**Supplementary Fig. 11**). However, several important nuances complicate this simplistic model.

Even if G protein biased agonists avoid G $\beta\gamma$ -independent GRK2 recruitment, G protein activation will still liberate G $\beta\gamma$, which in turn recruits GRK2 and partially suppresses G protein signaling (**Supplementary Fig. 11**), undercutting the intended bias. In addition, inhibiting basal receptor-GRK2 interactions could enhance G protein bias (**Supplementary Fig. 11**), particularly for receptors with strong constitutive GRK2 association. Conversely, arrestin biased ligands that rely on G $\beta\gamma$ -independent GRK2 recruitment may only partially elicit arrestin engagement and fail to drive receptor internalization (**Supplementary Fig. 11**). However, they could also facilitate more sustained arrestin recruitment compared to compounds that depend on G $\beta\gamma$ -dependent GRK2 recruitment and potentiate ERK signaling (**Supplementary Fig. 11**). For these reasons, biased agonists should be designed to have GRK2 recruitment profiles that match the therapeutic demand.

Third, G $\beta\gamma$ -independent GRK2 recruitment is both underleveraged by existing drugs and difficult to engage pharmacologically. None of the D2R compounds we tested — including balanced and biased agonists — fully activated this pathway compared to dopamine, underscoring its poor tractability with conventional chemotypes. This suggests that G $\beta\gamma$ -independent GRK2 recruitment may require receptor conformations that are difficult to stabilize. Thus, directly screening for GRK2 recruitment pathway-specific activity will be necessary to identify chemotypes capable of selectively engaging G $\beta\gamma$ -independent recruitment.

Lastly, uncovering and exploiting alternative GPCR binding sites could considerably advance biased agonist design. Most biased agonists act at the orthosteric binding site⁷³, with the expectation that specific active conformations preferentially engage the desired transducer. However, our data indicate that GRK2 can bind alternative sites that may be resistant to orthosteric modulation (e.g., at mGluR1). This suggests that allosteric modulators designed to occlude or enhance discrete intracellular receptor-transducer interfaces, including but not limited to that with GRK2, may offer a more effective route to highly biased agonists.

Methods

Cell lines

Human embryonic kidney 293T (HEK293T) were originally obtained from the American Type Culture Collection (ATCC, CRL-3216). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; high glucose) supplemented with 10% (v/v) fetal bovine serum (FBS) at 37°C in a humidified incubator with 5% CO₂. Cells were passaged every 2–3 days using trypsin-EDTA and were used between passages 5 and 40. Cell line identity was confirmed by the supplier. HEK293T cells are of human origin. Sex is not specified by the supplier and is not considered biologically relevant for the experiments performed.

GRK2/3/5/6 and Arr2/3 knockout cells (Δ Q:Arr2/3) were maintained in Dulbecco's modified Eagle's medium (DMEM; high glucose) supplemented with 10% (v/v) fetal bovine serum (FBS) at 37°C in a humidified incubator with 5% CO₂. Cells were passaged every 2–3 days using trypsin-EDTA and were used between passages 5 and 40. Sex is not specified and is not considered biologically relevant for the experiments performed.

Modeling

All molecular models were prepared based on published structures and/or those generated by AlphaFold3.0⁷⁴ using UCSF ChimeraX, which was developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco, with support from National Institutes of Health R01-GM129325 and the Office of Cyber Infrastructure and Computational Biology, National Institute of Allergy and Infectious Diseases.

Molecular Biology and Heterologous Expression

All constructs were cloned into mammalian expression vectors and are available from the authors upon request. HEK293T or ΔQ :Arr2/3 cells were maintained in DMEM (Invitrogen) with 10% fetal bovine serum at 37°C under 5% CO₂. For the BRET-based assays used in this study, cells were seeded at 200K to 400K in 24-well plates and transfected with Lipofectamine 2000 (1 μ L/well) and then washed and harvested in DPBS the following day. For electrophysiology experiments, cells were seeded in 15 mm coverslips that were placed in 24-well plates, transfected with Lipofectamine 2000 (1 μ L/well), and assayed the following day.

BRET-based assays

Cells used for the various BRET-based assays in this study were prepared and assayed as described²⁶. Briefly, cells were washed, harvested, and resuspended in DPBS at room temperature. Cells were distributed into a white 96-well microplate (Greiner). After incubation with coelenterazine H (5 μ M) or furimazine (5 μ M), various ligands were injected (see Figure Legends for further detail). Concentrations used in each experiment are described in Figure Legends. Using a Clariostar Plus plate reader (BMG Labtech), BRET¹ signal was determined by quantifying and calculating the ratio of the light emitted by a mVenus or citrine acceptor (535 nm $\pm$ 30 nm) over that emitted by an Nluc or Rluc8 donor (475 nm $\pm$ 30 nm).

Electrophysiology

ΔQ :Arr2/3 cells were sparsely seeded and maintained in DMEM (Invitrogen) with 10% fetal bovine serum on poly-L-lysine-coated coverslips at 37°C and 5% CO₂. ΔQ :Arr2/3 cells were voltage clamped in whole-cell configuration 16–48 hours after transfection. The extracellular solution contained 120 mM KCl, 25 mM NaCl, 10 mM HEPES, 2 mM CaCl₂, and 1 mM MgCl₂, pH 7.4. Glass pipettes with a resistance of 3–5 M Ω were filled with intracellular solution containing 120 mM Gluconic acid δ -lactone, 15 mM CsCl, 10 mM BAPTA, 10 mM HEPES, 1 mM CaCl₂, 3 mM MgCl₂, 3 mM MgATP, pH 7.2. Cells were voltage clamped to -80 mV using an Axopatch 200B amplifier (Molecular Devices). 10 μ M dopamine was applied using a gravity perfusion system. The selection criteria for electrophysiological experiments are that a cell (i) expresses the fluorescent protein transfection marker, and (ii) responds to agonist, indicating the presence of both receptor and GIRK. Cells were not excluded unless the recording was of poor quality (e.g., unstable baseline).

Data analysis

All experiments were performed in triplicate or quadruplicate and repeated at least three times. Electrophysiology data were analyzed using ClampFit (Molecular Devices). For dose–response curves, nonlinear regression analysis was performed using the sigmoidal dose–response function in GraphPad Prism. All values reported are mean $\pm$ standard error mean (S.E.M.). Statistical analyses were performed using GraphPad Prism 10 and are reported in Figure Legends.

Data availability

All data are available within the main text or the supplementary materials.

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Author contributions

Conceptualization: E.M and P.D. Experimentation: E.M, M.N, K.M., J.O., and P.D. Data Analysis: E.M, M.N., and P.D. Cell line development: J.D. and C.H. Manuscript Preparation: E.M. and P.D. Funding acquisition: P.D. All authors have given approval to the final version of the manuscript.

Competing Interests

The authors declare no competing interests.

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Figures

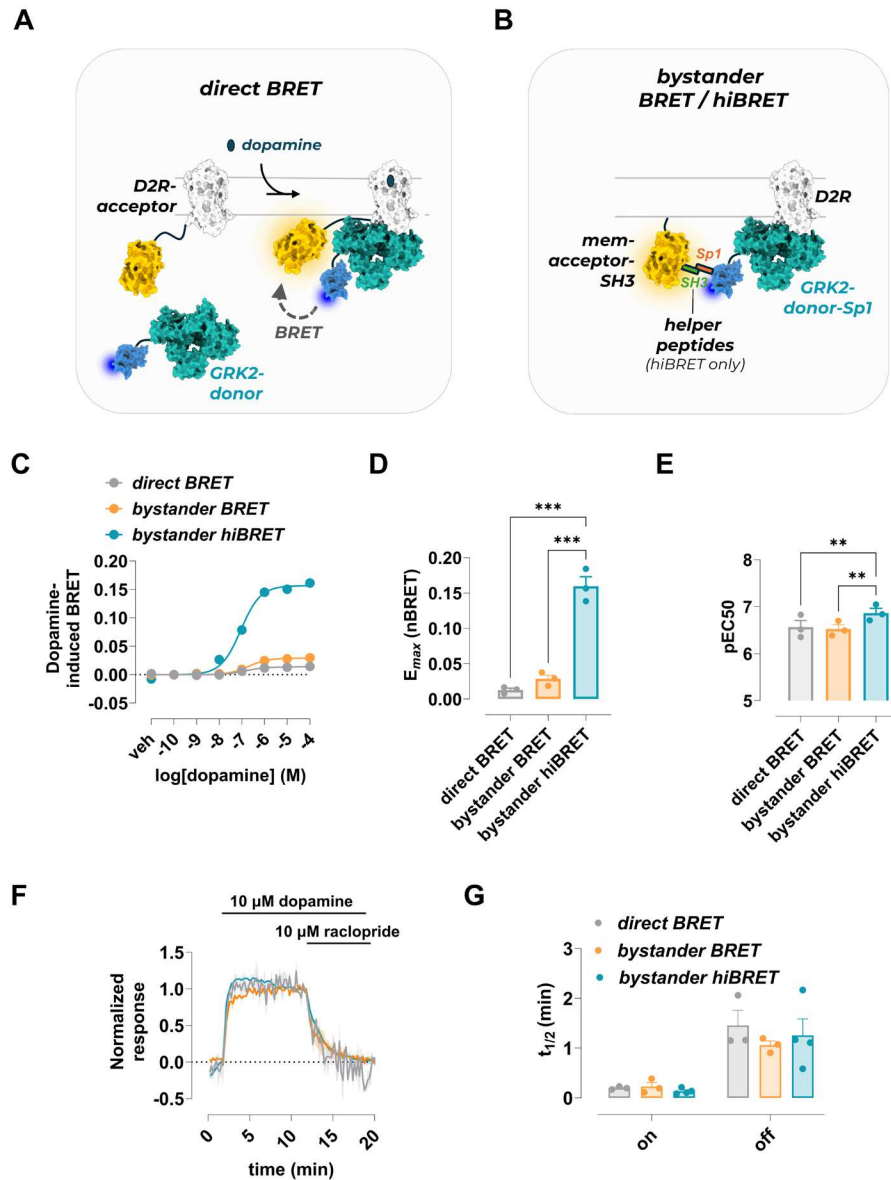


Figure 1. Development of a GRK2 bystander hiBRET assay for D2R. (A) A BRET assay between NanoLuc donor-tagged GRK2 (GRK2-donor) and mVenus acceptor-tagged D2R (D2R-acceptor) (direct BRET). (B) A BRET assay wherein unmodified D2R is co-expressed with GRK2-donor and a membrane-anchored citrine acceptor. This assay can be performed without modification to the donor and acceptor (bystander BRET) or with helper peptide Sp1 attached to GRK2-donor and helper peptide SH3 attached to the membrane-anchored acceptor (bystander hiBRET assay). (C) Representative dopamine dose-response curves in the D2R-dependent GRK2 recruitment direct BRET, bystander BRET, and bystander hiBRET assays. (D, E) Summary of dopamine E_{max} (D) and pEC_{50} (E) in the direct BRET, bystander BRET, and bystander hiBRET assays. RM one-way ANOVA, $F = 4.8$ for (D) and 50.0 for (E), Tukey's post-hoc test, *** $p < 0.001$, ** $p < 0.01$. (F) Representative traces of GRK2 recruitment to D2R in the direct BRET, bystander

BRET, and hiBRET assays. GRK2 recruitment was stimulated with 10 μ M dopamine and inhibited with 10 μ M raclopride. (**G**) Summary of the on and off kinetics of GRK2 recruitment in the direct BRET, bystander BRET, and bystander hiBRET assays. one-way ANOVA, $F = 1.1$ for on-kinetics and 0.4 for off-kinetics, Tukey's post-hoc test, no significant differences.

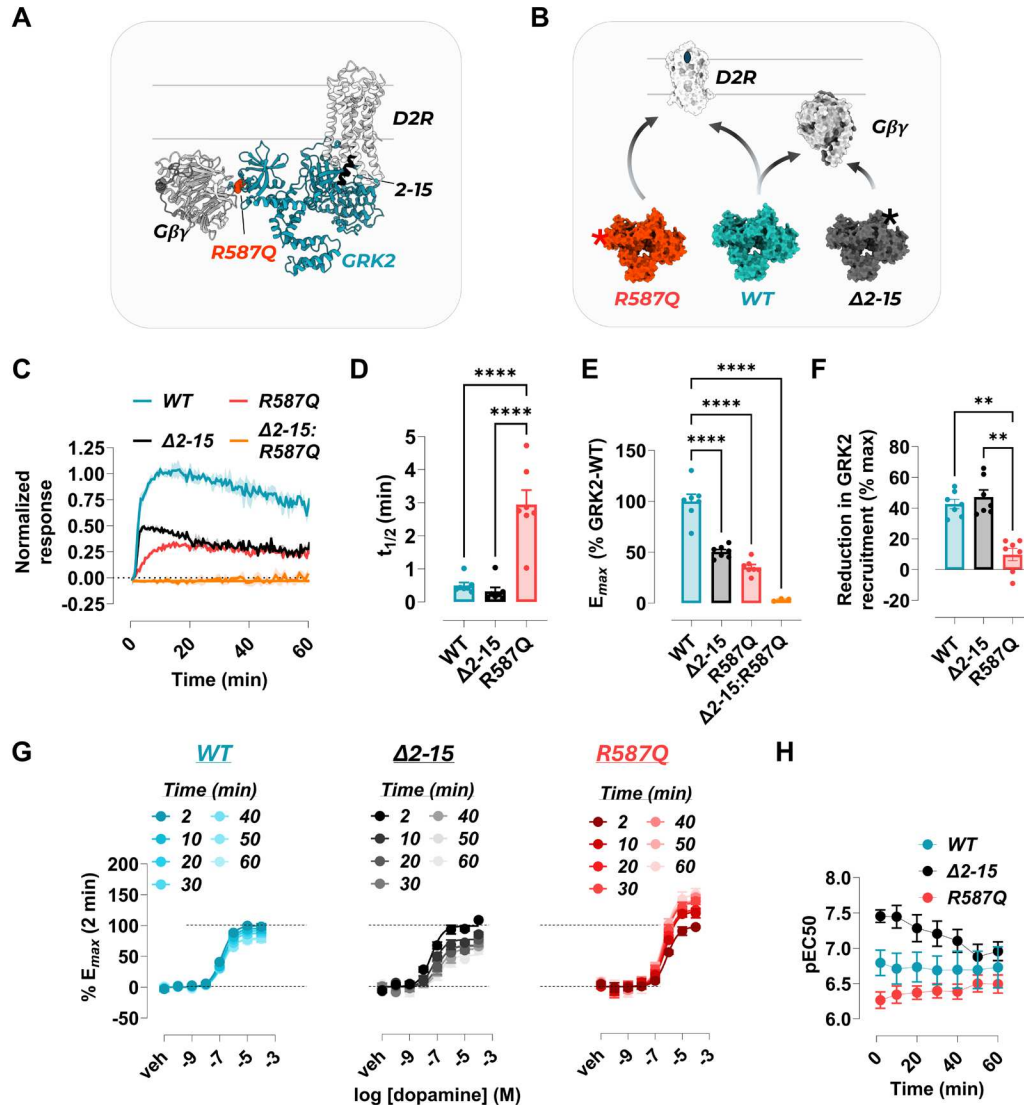


Figure 2. Dynamics of Gβγ-dependent and -independent GRK2 recruitment. (A) A molecular model of D2R in complex with GRK2 bound to Gβγ. GRK2 residues 2-15 bind the transmembrane bundle of the receptor, whereas R587 interacts with Gβγ. (B) A mutant of GRK2 that lacks its N-terminus (Δ2-15) binds Gβγ but not D2R, whereas GRK2 mutant R487Q binds D2R but not Gβγ. (C) Representative traces of the recruitment of GRK2-WT, GRK2(Δ2-15), GRK2(R587Q), or GRK2(Δ2-15:R587Q) to the plasma membrane in response to 10 μM dopamine-induced D2R activation. (D) Maximal recruitment of GRK2-WT or mutant GRK2s to the plasma membrane. one-way ANOVA, $F = 65.5$, **** $p < 0.0001$. (E) Summary of the on-kinetics of GRK2-WT, GRK2(Δ2-15), or GRK2(R587Q) to the plasma membrane in response to 10 μM dopamine. RM one-way ANOVA, $F = 32.7$, **** $p < 0.0001$. (F) Reduction in maximal GRK2 recruitment to the plasma membrane after 60 minutes. RM one-way ANOVA, $F = 22.4$, ** $p < 0.01$. (G) GRK2-WT, GRK2(Δ2-15), or GRK2(R587Q) recruitment in response to increasing concentrations of dopamine over time. (H) Summary of the potency of dopamine for GRK2-WT, GRK2(Δ2-15), or GRK2(R587Q) over time. $n = 3$ to 4 replicates per condition.

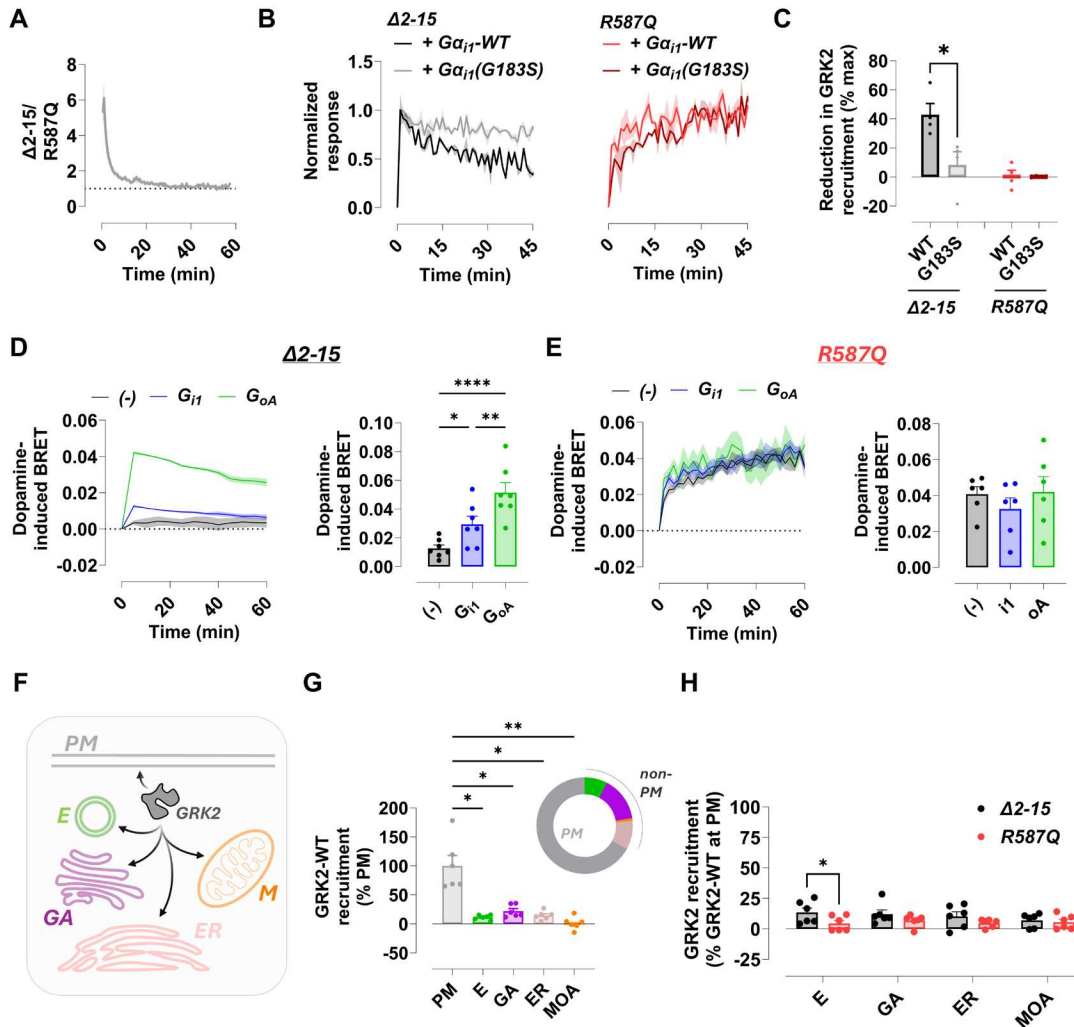


Figure 3. G protein-dependence of G $\beta\gamma$ -dependent and -independent GRK2 recruitment. (A) Relative difference in D2R-mediated GRK2($\Delta 2-15$) or GRK2(R587Q) recruitment over time. (B,C) Recruitment of GRK2($\Delta 2-15$) (C) or GRK2(R587Q) (D) in response to 100 μ M dopamine in the presence of PTX and either PTX-resistant $G\alpha_{i1}$ -WT or $G\alpha_{i1}(G183S)$. (D) Representative traces (left) and summary (right) of 100 μ M dopamine-induced GRK2($\Delta 2-15$) recruitment to the plasma membrane in the presence of PTX (-) and either PTX-resistant $G\alpha_{i1}$ or $G\alpha_{oA}$. RM one-way ANOVA, $F = 21.9$, Tukey post-hoc test, * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$. (E) Representative traces (left) and summary (right) of 100 μ M dopamine-induced GRK2(R587Q) recruitment to the plasma membrane in the presence of PTX and either PTX-resistant $G\alpha_{i1}$ or $G\alpha_{oA}$. RM one-way ANOVA, $F = 1.6$, Tukey post-hoc test, no significant differences. (F) Schematic of GRK2 recruitment to various organelles, including the plasma membrane (PM), endosomes (E), Golgi apparatus (GA), endoplasmic reticulum (ER), and mitochondria (M). (G) Summary of D2R-dependent GRK2-WT recruitment to various organelles in response to 1 mM dopamine. RM one-way ANOVA, $F = 20.8$, Tukey post-hoc test, * $p < 0.05$, ** $p < 0.01$. Inset shows the proportion of GRK2-WT recruitment to various intracellular compartments. (H) Summary of the preference of each compartment for GRK2($\Delta 2-15$) or GRK2(R587Q) in response to D2R activation. two-way ANOVA, $F = 20.8$, Tukey post-hoc test, * $p < 0.05$.

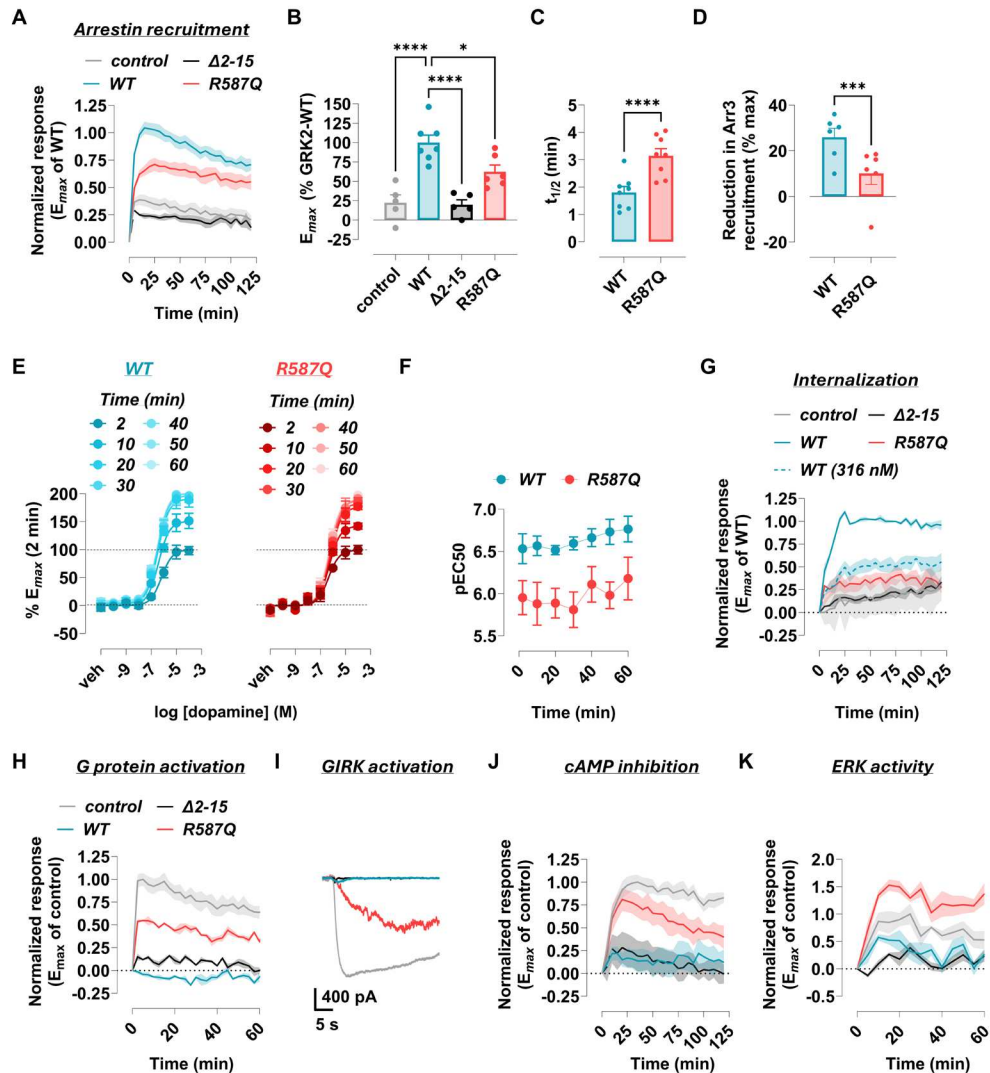


Figure 4. Differential effects of G β γ -dependent and -independent GRK2 recruitment on D2R signaling. (A) Representative trace of Arr3 recruitment to D2R in Δ Q:Arr2/3 cells overexpressing GRK2-WT, GRK2(Δ 2-15), or GRK2(R587Q) in response to 100 μ M dopamine. (B) Summary of Arr3 recruitment to D2R in Δ Q:Arr2/3-GRK cells. one-way ANOVA, $F = 18.4$, Tukey post-hoc test, $*p < 0.05$, $****p < 0.0001$. (C) Summary of the on-kinetics of Arr3 recruitment in the presence of GRK2-WT, GRK2(Δ 2-15), or GRK2(R587Q). paired t-test, $****p < 0.0001$. (D) Reduction in maximal Arr3 recruitment after 100 minutes. paired t-test, $***p < 0.001$. (E) Representative dopamine dose-response curves of Arr3 recruitment to D2R. (F) Summary of the potency of dopamine-induced Arr3 over time. two-way ANOVA, $F = 49.2$, $****p < 0.0001$. $n = 3$ replicates per condition. (G) Representative traces of D2R internalization in the presence of GRK2-WT, GRK2(Δ 2-15), or GRK2(R587Q) compared to control in Δ Q:Arr2/3-GRK cells. Arr3 was co-expressed in all conditions. 100 μ M dopamine was applied to all conditions, with an additional condition in which 316 nM dopamine was applied to GRK2-WT. (H) Representative traces of D2R-dependent G protein activation the presence of GRK2-WT, GRK2(Δ 2-15), or GRK2(R587Q) compared to control in Δ Q:Arr2/3-GRK cells. Arr3 was co-expressed in all conditions. 100 μ M dopamine was applied to all conditions (I) Representative traces of D2R-dependent GIRK activation the presence of GRK2-WT, GRK2(Δ 2-15), or GRK2(R587Q) compared to control in

Δ Q:Arr2/3 cells. Arr3 was co-expressed in all conditions. 10 μ M dopamine was applied to all conditions (**J**) Representative traces of D2R-dependent cAMP inhibition the presence of GRK2-WT, GRK2(Δ 2-15), or GRK2(R587Q) compared to control in Δ Q:Arr2/3 cells. Arr3 was co-expressed in all conditions. 100 μ M dopamine was applied to all conditions. (**K**) Representative traces of D2R-dependent ERK activity in the presence of GRK2-WT, GRK2(Δ 2-15), or GRK2(R587Q) compared to control in Δ Q:Arr2/3 cells. Arr3 was co-expressed in all conditions.

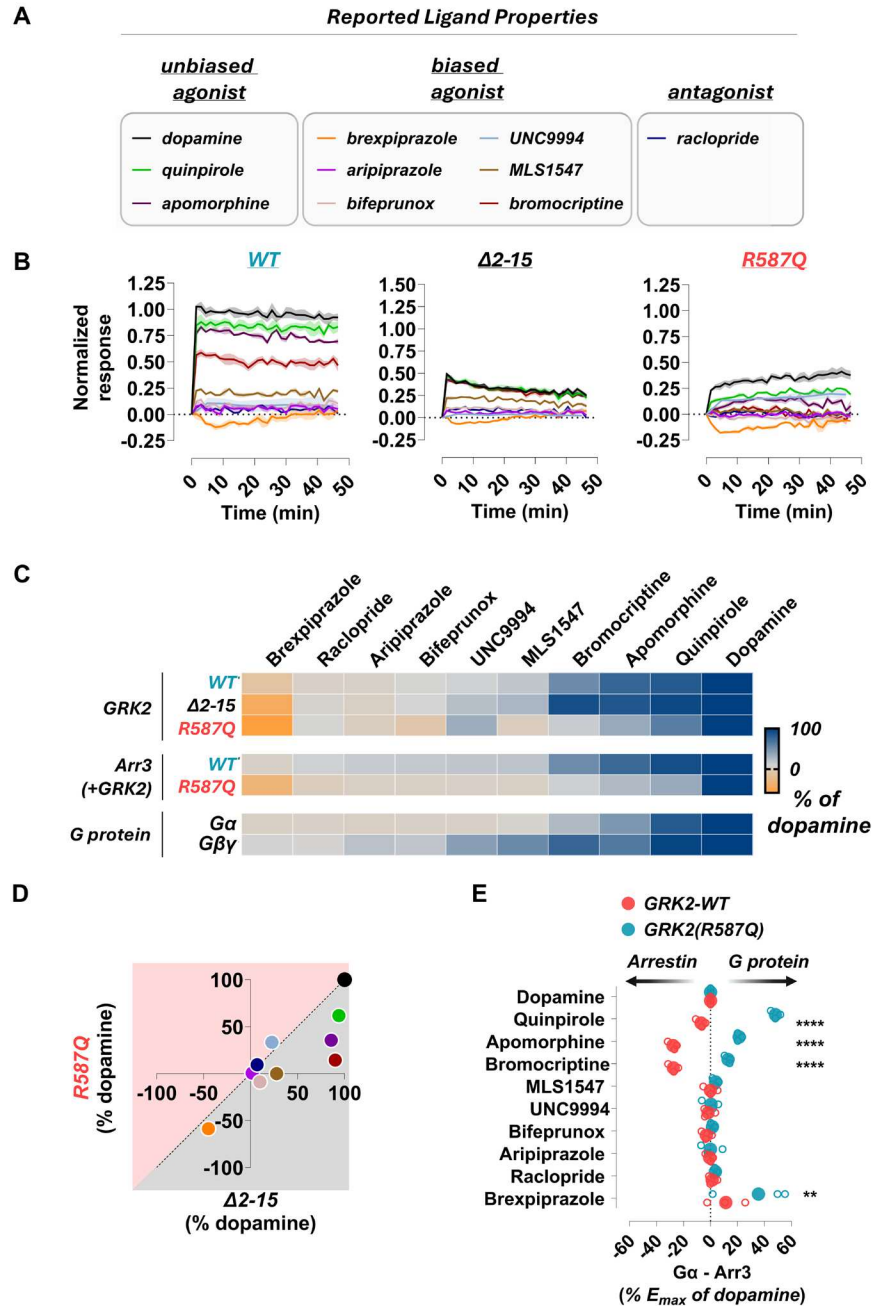


Figure 5. D2R ligands differentially promote G $\beta\gamma$ -dependent and -independent GRK2 recruitment. (A) D2R agonists and antagonists characterized in this study categorized based on their *reported* pharmacological properties. (B) Representative traces of GRK2-WT (left), GRK2(Δ 2-15) (middle), or GRK2(R587Q) (right) recruitment in response to a saturating concentration of various compounds. (C) Summary of D2R ligand-dependent effects on GRK2-WT and its mutants, G protein, and arrestin. All synthetic ligands were applied at 10 μ M, and responses are normalized to saturating dopamine (100 μ M) within each assay. (D) Plot of the preference of each compound for GRK2(Δ 2-15) or GRK2(R587Q). Equipotent activity for GRK2(Δ 2-15) and GRK2(R587Q) falls on a linear reference line with a slope of 1. (E) D2R ligand preference for G protein or arrestin when arrestin recruitment is driven by GRK2-W or GRK2(R587Q). two-way ANOVA, $F = 75.1$, Sidak's post-hoc test, ** $p < 0.01$, **** $p < 0.0001$.

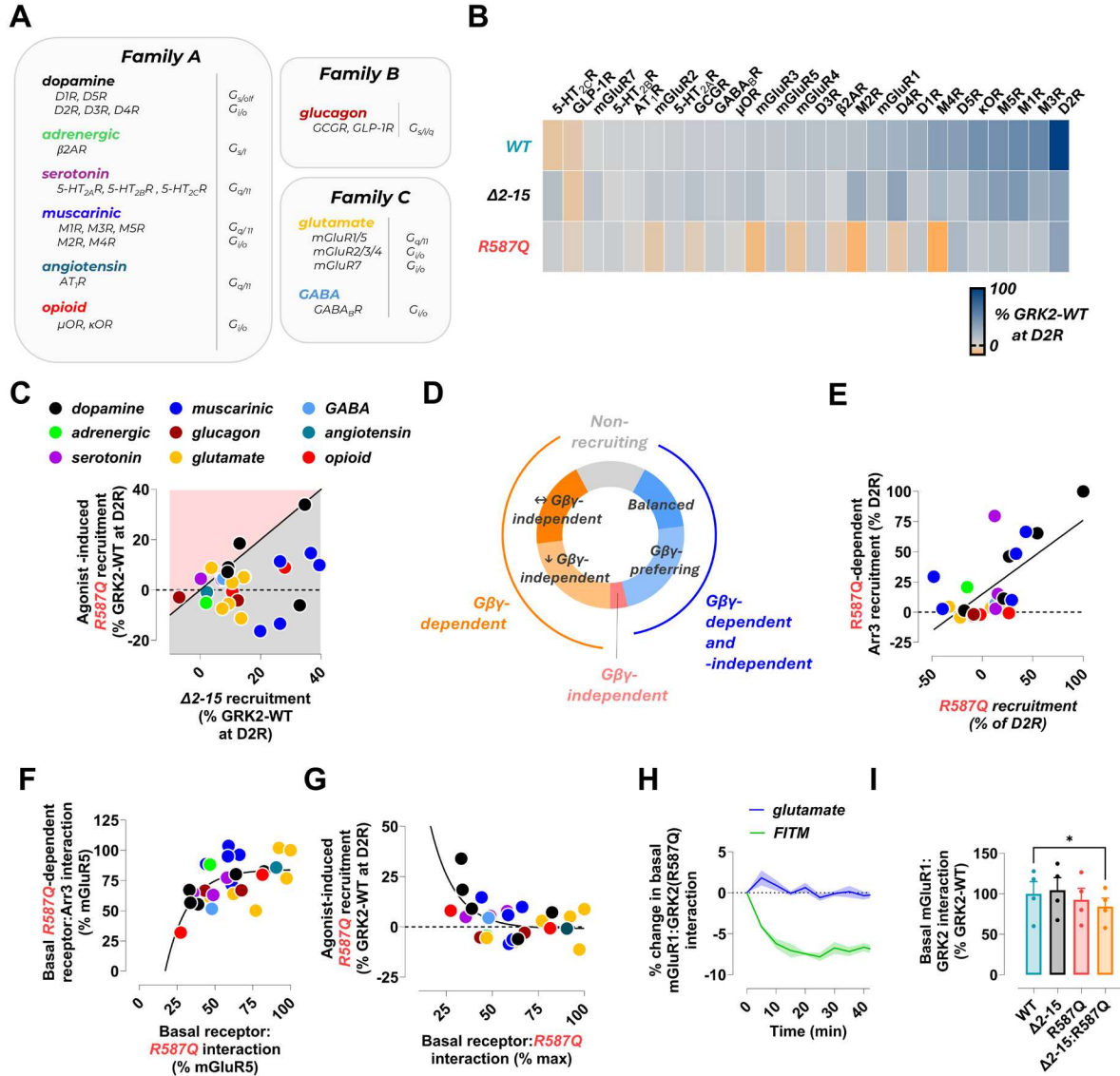


Figure 6. GPCRs differentially promote $G\beta\gamma$ -dependent and -independent GRK2 recruitment. (A) GPCRs screened in the GRK2 bystander hiBRET assay divided by family and G protein coupling preference. (B) Summary of GRK2-WT, GRK2(Δ 2-15), or GRK2(R587Q) recruitment to various GPCRs ranked from lowest to highest, shown as a percentage of GRK2-WT recruitment to D2R. (C) Plot of the preference of each GPCR for GRK2(Δ 2-15) or GRK2(R587Q). Equipotent activity for GRK2(Δ 2-15) and GRK2(R587Q) falls on a linear reference line with a slope of 1. (D) GPCRs categorized based on their GRK2 recruitment profile. (E) Relationship between GRK2(R587Q)-dependent Arr3 recruitment and GRK2(R587Q) recruitment across GPCRs. Linear regression, Slope = 0.61, $R^2 = 0.27$. (F) Relationship between basal GRK2(R587Q)-dependent:Arr3 interaction and basal receptor:GRK2(R587Q) interaction. Non-linear regression, $Sy.x = 38.3$ (G) Relationship between agonist-induced GRK2(R587Q) recruitment and the basal receptor:GRK2(R587Q) interaction. Non-linear regression, $Sy.x = 11.9$. (H) Representative trace of the effect of glutamate (1 mM) or FITM (10 μ M) on the interaction between mGluR1 and GRK2(R587Q). (I) Summary of the basal interaction between mGluR1 and GRK2-WT, GRK2(Δ 2-15), GRK2(R587Q), or GRK2(Δ 2-15:R587Q). RM one-way ANOVA, $F = 9.0$, Tukey post-hoc test, $*p < 0.05$.

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