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Direct interrogation of context-dependent GPCR activity with a universal biosensor platform

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SUMMARY

G protein-coupled receptors (GPCRs) are the largest family of druggable proteins encoded in the human genome, but progress in understanding and targeting them is hindered by the lack of tools to reliably measure their nuanced behavior in physiologically-relevant contexts. Here, we developed a collection of compact ONE vector G-protein Optical (ONE-GO) biosensor constructs as a scalable platform that can be conveniently deployed to measure G-protein activation by virtually any GPCR with high fidelity even when expressed endogenously in primary cells. By characterizing dozens of GPCRs across many cell types like primary cardiovascular cells or neurons, we revealed insights into the molecular basis for G-protein coupling selectivity of GPCRs, pharmacogenomic profiles of anti-psychotics on naturally-occurring GPCR variants, and G-protein subtype signaling bias by endogenous GPCRs depending on cell type or upon inducing disease-like states. In summary, this open-source platform makes the direct interrogation of context-dependent GPCR activity broadly accessible.

Graphical Abstract

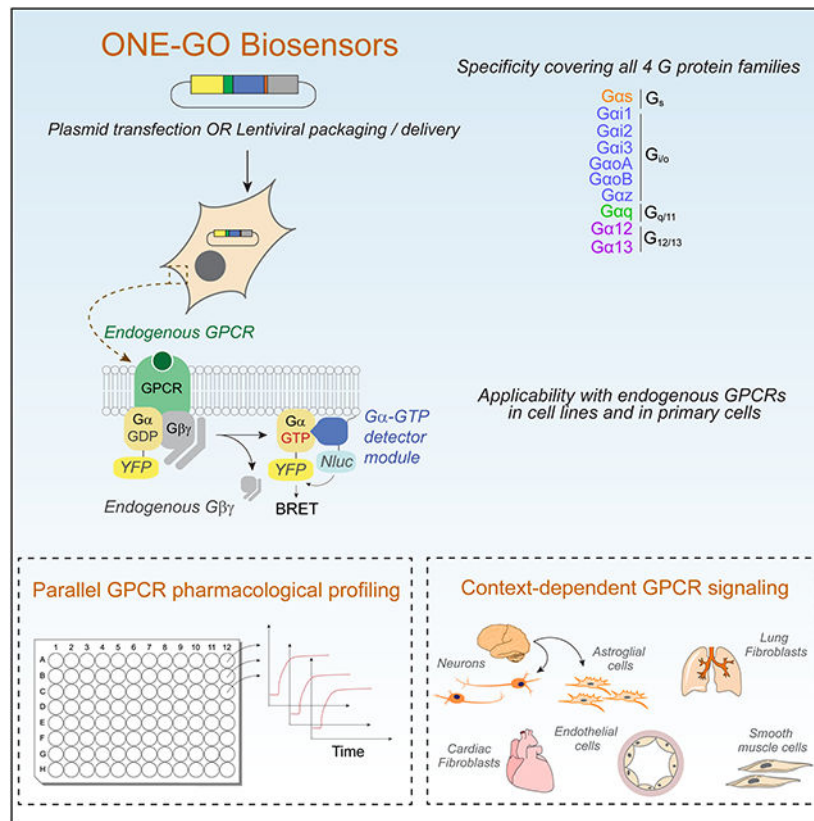
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In Brief

Development and implementation of a suite of G protein biosensors broadly applicable to detect GPCR activity in scalable assay formats and in physiologically-relevant systems like primary cells. By directly measuring endogenous GPCR activity, these biosensors, named ONE-GO, reveal that responses are frequently dependent on the cell context or state.

INTRODUCTION

G-protein-coupled receptors (GPCRs) mediate responses to many natural stimuli, including most neurotransmitters and two-thirds of hormones^{1–3}, and are also the target for 30–40% of clinically-approved drugs^{4,5}. While representing the largest family of druggable targets encoded in the human genome, there is still vast untapped potential because the majority of GPCRs remain understudied⁶. With the advent of wide-spread success in elucidating high resolution structures of many GPCRs^{7,8}, current trends in GPCR research and drug development are focused on identifying mechanisms and compounds that fine tune signaling outcomes, including allosteric modulation or ligand-directed biased signaling^{9–12}. However, progress in this area has been hampered by the overreliance on assays that measure GPCR activity indirectly (e.g., second messengers), and on a limited number of cell lines. As a consequence, pharmacological features of compounds characterized by *in vitro* GPCR assays may not accurately translate to the expected therapeutic benefit *in vivo*¹³, as illustrated by ongoing controversies on experimental opioid drugs with diminished side

effects^{14–19}. A critical factor for the discrepancy between *in vitro* and *in vivo* pharmacology is the existence of *system biased GPCR signaling*, i.e., how signaling pathways are hardwired in each specific cellular system to propagate the initial drug-receptor interaction to a cellular response^{13,20}.

An approach to mitigate the impact of system bias in drug discovery is the use of assays that measure GPCR activity more directly to avoid potential crosstalk and amplification mechanisms specific to a given cellular system. In this regard, GPCRs propagate signaling primarily by directly activating heterotrimeric G proteins ($G\alpha\beta\gamma$), although some responses are mediated by β -arrestins^{1,2,21}. GPCRs activate G proteins by promoting the exchange of GDP for GTP on $G\alpha$ subunits. In turn, this leads to the dissociation of $G\alpha$ -GTP and free $G\beta\gamma$ ^{22,23}. The latter event has been widely exploited to generate biosensors of G protein activity based on fluorescence or bioluminescence resonance energy transfer (FRET or BRET, respectively)^{24–28}. While $G\alpha$ / $G\beta\gamma$ dissociation is an event proximal to GPCR activation, these approaches are not broadly applicable to physiologically relevant systems and can significantly compromise the fidelity of the responses measured. For example, even in optimized versions of biosensors that measure $G\alpha$ / $G\beta\gamma$ dissociation (e.g., TRUPATH²⁹), overexpression of exogenous G protein subunits tagged with BRET donors and acceptors is required. This imposes several limitations. One is that multiple genetic components must be delivered simultaneously to cells in optimal proportions, making the approach only feasible in a few easily transfectable cell lines. Another major limitation is that exogenous G protein subunits can form non-BRET productive complexes with endogenous G proteins, so they must be expressed in large excess to yield robust BRET responses. This represents a significant perturbation of the system under investigation by the method of detection employed.

We recently developed alternative G protein biosensor designs to measure $G\alpha$ -GTP in cells as a more proximal event to receptor activation than $G\alpha$ / $G\beta\gamma$ dissociation, and converted them into single polypeptide chain biosensors named BERKY³⁰. While this allowed the detection of endogenous GPCR activity in primary neurons and without interfering with downstream signaling³⁰, the small dynamic range has remained a limitation for the broad applicability of this type of biosensor. Others have developed an effector membrane translocation assay (EMTA) that also measures $G\alpha$ -GTP³¹. However, some EMTA biosensors are likely to interfere with G protein signaling, and the broad applicability of EMTA with endogenous GPCRs in primary cells has not been established, probably because of the need to deliver simultaneously multiple genetic components. A notable exception for both BERKY and EMTA biosensor systems is the lack of direct detection of $G\alpha$ -GTP species for one of the four G protein families, G_s ²².

In the work presented here, we generated a comprehensive collection of $G\alpha$ -GTP biosensor constructs for different G protein types, including the evasive G_{as} -GTP, that combines large dynamic range of detection with the ability to monitor endogenous GPCR activity across a wide range of physiologically-relevant systems (e.g., different primary cell types). By leveraging the advantages of this biosensor platform, we reveal that endogenous GPCRs in primary cells display unique signaling behaviors depending on the cellular context, raising the important point that system bias must be considered even when measuring direct

readouts of GPCR signal transduction. To facilitate adoption and customization by others, all the biosensor constructs described here and their designs have been made publicly accessible through Addgene as an open-source platform.

RESULTS

Direct detection of active Gas in cells with a BRET biosensor

We sought to identify a protein sequence that would bind specifically to active Gas, anticipating that it would serve as a specific detector module for a genetically encoded live-cell biosensor. Initial efforts based on previously described Gas interactors^{32–34} failed to identify a suitable detector module (*not shown*). We turned our attention to the patent literature and found a series of putative Gas-GTP binding peptides from a phage display screen (Fig.1A³⁵). We tested 12 of these peptides for binding to active Gas (Fig.1A) and determined that sequences KB1691 and KB2123 bound strongly to active Gas, but not active Gαo (Fig.1A). Then, we fused KB1691 or KB2123 to membrane-anchored nanoluciferase (Nluc, BRET donor) and co-expressed each one of them in HEK293T with Venus-tagged Gas (BRET acceptor), along with Gβ₁, Gγ₂, and the β₂ adrenergic receptor (β₂AR) (Fig.1B, Fig.S1). We systematically tested three versions of Gas (short isoform) in which Venus was inserted at different internal locations³⁶, with and without co-expression of the Gas chaperone Ric-8B³⁷. Both KB1691- and KB2123-based sensors performed best with the Gas constructs with Venus inserted at codon 99 (Gas-99V) and in the presence of Ric-8B— i.e., these conditions led to larger BRET increases upon stimulation with the β-adrenergic agonist isoproterenol, which were reversed upon treatment with the antagonist alprenolol (Fig.1B, Fig.S1A–B). Similar results were obtained with a second GPCR, the dopamine D1 receptor (D1R) (Fig.S1C). Amino acid position 99 is in a loop of the helical domain of Gas that tolerates insertion of bulky tags, as previously demonstrated by the functional validation of Gas-99V and other constructs with insertions in the same loop^{25,38}. While Ric-8B expression improved the magnitude of the responses detected, this was not due to a significant increase in Gas-99V expression (Fig.S1A–B). This suggests that the improved responses are due to better folding and/or membrane targeting of tagged Gas, as previously reported for Ric-8 proteins by others³⁷. An alternative biosensor design relying on bystander BRET^{39,40} was also attempted (Fig.S1D), in part because no Gas-GTP sensor has been designed based on this principle due to the lack of adequate detector modules for it^{31,41}. Upon GPCR stimulation, cytosolic Nluc-fused KB1691 peptide was recruited to active, untagged Gas at the plasma membrane, where it yielded BRET with a bystander YFP anchored in that location (Fig.S1D). However, the response was not as robust as with the system composed of the membrane-anchored, Nluc-fused peptide and Gas-99V in the presence of Ric-8B, which became the biosensor design of choice for subsequent studies. These results indicate that GPCR-mediated activation of Gas can be detected in cells with an optical biosensor based on peptides that bind Gas-GTP.

Specific detection of active Gas but no other G protein type

Next, we asked if the identified biosensors were specific for Gas. To test this, we replaced Gas-99V with YFP-tagged Gα subunits representative of the other three G protein families (i.e., Gαi3 for G_{i/o}, Gαq for G_{q/11}, and Gα13 for G_{12/13}), and measured BRET responses

upon stimulation of cognate GPCRs (Fig.1C). Activation of Gai3, Gaq and Gα13 under these conditions was confirmed in parallel experiments with previously validated biosensors for each one of these G proteins³⁰ (Fig.S1E). We found that KB1691-based sensors only detected activation of Gas, whereas KB2123-based sensors detected activation of both Gas and Gai3 (Fig.1C). These results demonstrate that biosensors based on the KB1691 sequence, but not on the KB2123 sequence, specifically detect activation of Gas. For this reason, we focused our subsequent efforts on KB1691-based biosensors.

Gas-GTP biosensor does not interfere with downstream signaling

A potential concern of any biosensor is the potential interference with the process it measures. Because Nluc is a bright luciferase that can be expressed at low levels to obtain measurable luminescence signals⁴², we reasoned that expression of the biosensor with Nluc-fused KB1691 would not interfere significantly with G_s signaling in cells. To test this, we measured cAMP production upon β2AR stimulation in HEK293T under conditions identical to those used to detect Gas activation with this sensor, except that a probe for cAMP⁴³ was co-expressed. We found that the cAMP response detected was not affected by the expression of the Gas BRET sensor (Fig.1D), indicating no overt effect on GPCR-stimulated G_s signaling to downstream effectors.

Gas-GTP biosensor reveals specific activation properties of oncogenic Gas mutants

Having established a biosensor for Gas-GTP, we set out to leverage it in functional studies, starting with characterizing the activation properties of cancer-associated G protein mutants. GNAS, the gene encoding Gas, is frequently mutated in cancer at two hotspots corresponding to R201 and Q227 in the protein^{44,45}. These mutations lead to Gas forms that are constitutively active in cells— i.e., they elevate cAMP levels under unstimulated conditions⁴⁶. Both residues participate in GTP hydrolysis (Fig.2A), suggesting that the mechanism by which their mutation leads to constitutive signaling is by locking them in a GTP-bound state. However, *in vitro* results with purified proteins suggest that Q227 mutants are likely to be completely occupied by GTP, whereas R201 mutants might be only partially occupied with GTP^{47,48}. Whether this is the case in cells is not known, so we leveraged the Gas-GTP biosensor developed by us for this purpose. We found that, under unstimulated conditions, Gas R201C gave a higher BRET signal than Gas WT, yet it was much lower than the BRET signal for Gas Q227L (Fig.2A). Moreover, isoproterenol stimulation did not lead to an increase of the BRET signal for Gas Q227L (Fig.2A), consistent with the interpretation that this mutant is completely occupied with GTP in cells and cannot be further activated. In contrast, isoproterenol stimulation led to an increase of BRET for Gas R201C, which was not reverted upon an addition of antagonist that fully reverted the isoproterenol response with Gas WT (Fig.2A). While the latter indicates that Gas R201C is indeed GTPase-deficient, the former suggests that Gas R201C is susceptible to further GTP loading upon acute receptor stimulation. Taken together, these results indicate that, as opposed to Gas Q227L, Gas R201C is only partially occupied with GTP in cells.

Gas-GTP biosensor reveals G_s structural determinants for productive GPCR coupling

Given that the G_s activity biosensor we developed measures the direct product of the catalytic GEF activity of GPCRs, i.e., Gas-GTP, we set out to leverage it to characterize

structural determinants of G α s required for receptor-mediated activation. While it is well-established that the C-terminal tail of G α is critical for GPCR coupling, it has become clear that this is not the sole determinant for coupling^{49–52}. Based on the structure of the β 2AR-Gs complex and other related GPCR-G protein structures, the α N/ β 1 hinge region of G α s makes extensive contact with the receptor and may serve as an allosteric conduit between the receptors and the nucleotide binding pocket of G proteins^{53,54} (Fig.2B). To directly determine the relative contribution of the C-terminal tail (CT) and the α N/ β 1 hinge to the productive coupling of G α s to GPCRs, we used the G α s-GTP biosensor to measure β 2AR-mediated activation of G α s chimeras in which the CT and/or the α N/ β 1 hinge had been replaced by the corresponding sequences in G α i3, which is not activated by this receptor (Fig.2C, FigS2). As expected, replacement of the CT led to a marked decrease in activation (~50%) compared to wild-type (Fig.2C, Fig.S2). Interestingly, replacement of α N/ β 1 hinge led to a similar decrease in activation (~50%), and the effects became additive when both CT and α N/ β 1 hinge were replaced (~95% decrease). These results indicate that not only the CT, but also the α N/ β 1 hinge, is necessary for efficient coupling of G α s to β 2AR (Fig.2C, FigS2). Moreover, replacement of the α N/ β 1 hinge region impaired activation by four other GPCRs known to couple primarily to G α s (Fig.2C, FigS2), indicating that the requirement for the α N/ β 1 hinge region is a general feature of G α s-GPCR coupling. Similar experiments were carried out with reciprocal G α i3 chimeras to assess what elements of G α s are *sufficient* to permit activation. Replacement of the CT of G α i3 with the corresponding region of G α s led to some activation upon stimulation of the β 1AR and EP2 (Fig.2D, FigS2). When both the CT and α N/ β 1 hinge regions were replaced simultaneously, activation was enhanced for these two receptors compared to the CT chimera, and responses became detectable for two additional GPCRs (β 2AR and 5HT7) (Fig.2D, FigS2). These results highlight the importance of the α N/ β 1 hinge region of G α for activation by GPCRs that primarily couple to G α s. However, we found that this is not the case for GPCRs primarily coupled to G α i because replacement of the α N/ β 1 region of G α i3 by the corresponding region of G α s had little or no effect on its activation by four different receptors, while replacement of the CT completely ablated the responses (Fig.2D, FigS2). Moreover, grafting the α N/ β 1 hinge region of G α i3 into G α s was not sufficient to achieve activation of the non-cognate G protein by any of the four G α i-linked GPCRs, whereas replacement of the CT led to activation by three of them (Fig.2C, FigS2). These results indicate that the CT, but not α N/ β 1 hinge, of G α i has a prominent role in achieving productive coupling to GPCRs. Taken together, our observations indicate that the α N/ β 1 hinge region is a structural determinant specifically important for G α s productive coupling to cognate GPCRs, and that this feature might not be shared by other G proteins.

ONE vector G protein Optical (ONE-GO) biosensor designs display improved features

Next, we set out to condense all the components of the G α s-GTP biosensor into a single vector. We reasoned that delivery of the biosensor as a single payload would provide several advantages. For example, it would facilitate implementation in high-throughput formats or when investigating endogenous GPCRs in poorly transfectable primary cells. Another advantage of delivering the biosensor components as a single payload is that every cell receiving the construct would express all components required for the BRET biosensor^{55,56}, thereby increasing the magnitude of the responses detected while simultaneously reducing

the total amount of exogenous G protein required (Fig.3A). As an initial step, we reduced the number of components required for detection of Gas from five to three by removing exogenous expression of G β and G γ , which do not directly participate in the BRET interaction between Gas-99V and Nluc-fused KB1691 (Fig.3A, Fig.S3A). We found that expression of exogenous G $\beta\gamma$ was dispensable to obtain robust BRET responses (Fig.S3A). This not only indicates that we can reduce the number of components expressed without sacrificing quality of performance, but also that our biosensor detects responses of G protein heterotrimers assembled with endogenous G $\beta\gamma$.

We then set out to design and optimize a tricistronic Ga-GTP biosensor construct coding for Gas-99V, a membrane-anchored KB1691-Nluc, and Ric-8B (Fig.3A), which we named Gas ONE vector G protein Optical biosensor (Gas ONE-GO biosensor). This construct and its derivatives described below were assembled in a lentiviral backbone ensuring that the size of the payload remained below the limit for lentiviral packaging. We explored designs with two promoters of different strength, SV40 and CMV, in which Gas-99V was placed directly downstream of the promoter and KB1691-Nluc downstream of an IRES, followed by a self-cleaving T2A sequence and Ric-8B (Fig.S3B). We used a modified IRES with low efficiency (IRES*)^{57,58} to achieve a higher acceptor to donor expression ratio, which is expected to improve the dynamic range of BRET responses. Similar designs were also developed for another G protein (Gai3) using a cognate Ga-GTP detector module (KB1753 peptide^{30,59}), with the exception that no chaperone was included (Fig.S3B). To rigorously test the performance of the ONE-GO biosensors relative to their multi-plasmid (M-P) counterparts³⁰, we compared them side by side under identical experimental conditions. We found that ONE-GO biosensors led to larger responses upon GPCR stimulation (Fig.3B–C, Fig.S3B), especially for the designs incorporating the CMV promoter. Moreover, this improvement in performance was accompanied with much lower levels of expression of G protein and Nluc-fusions with ONE-GO compared to M-P (Fig.3B–C). In particular, G protein expression was barely detectable by immunoblotting, and lower than expression of the corresponding endogenous G protein. Overall, these results indicate that ONE-GO biosensors detect robust responses under near-endogenous G protein expression conditions, in which low amounts of exogenous Ga subunits assemble into functional heterotrimers with endogenous G $\beta\gamma$ dimers.

ONE-GO biosensors do not interfere with GPCR-G protein signaling

To mitigate potential concerns of interference of ONE-GO biosensors with GPCR signaling, we tested their effect on a signaling readout downstream of G protein activation (i.e., cAMP). We found that neither Gas ONE-GO nor Gai3 ONE-GO changed the efficacy or potency of GPCR-mediated cAMP responses (Fig.3D). To further rule out the potential interference of the G protein-binding modules in the biosensor with normal G protein function, we determined if expression of these sequences interfered with Ga-G $\beta\gamma$ resting association or their dissociation upon GPCR stimulation (Fig.S4A). Using a biosensor that measures free G $\beta\gamma$ levels in cells, we found that neither the KB1691 nor the KB1753 sequence, used in Gas ONE-GO and Gai3 ONE-GO, respectively, altered the association of G $\beta\gamma$ with Ga before or after GPCR stimulation (Fig.S4A).

ONE-GO biosensors report activation across G protein families and for many GPCRs

Next, we extended the ONE-GO biosensor design to allow the detection of different $G\alpha$ -GTP species across all four G protein families (G_s , $G_{i/o}$, $G_{q/11}$ and $G_{12/13}$) (Fig.4A). We implemented the single plasmid design described above without co-expression of a chaperone by pairing $G\alpha$ subunits tagged with YFP at the α b- α c loop of the all-helical domain, which tolerates insertions without affecting G protein function^{25,30,60}, with membrane-anchored Nluc-fused detector modules³⁰. We did not attempt to develop constructs for $G\alpha_{t1}$, $G\alpha_{t2}$, $G\alpha_{\text{gust}}$, $G\alpha_{\text{olf}}$, or $G\alpha_{15}$ because we lacked validated or suitable detector modules. We generated ONE-GO constructs for $G\alpha_{11}$ and $G\alpha_{14}$ using GRK2^{RH} as the detector module based on prior evidence⁶¹, but were not successful in detecting responses with them upon GPCR stimulation. In total, we validated ONE-GO biosensors for 10 different $G\alpha$ subunits (Fig.4A; all ONE-GO sensor sequences are provided in [SI1]), including representative members of all four G protein families. Analogous to what was observed for other ONE-GO biosensors for G proteins that regulate AC/cAMP signaling (Fig.3), we found that that expression of $G\alpha_q$ ONE-GO did not affect ERK1/2 phosphorylation downstream of PLC/ Ca^{2+} /PKC signaling (Fig.S4B).

To assess the broad utility of ONE-GO biosensors, we implemented them with a large panel of GPCRs and compared results with curated pharmacological parameters (e.g., K_i values) available through the International Union of Basic and Clinical Pharmacology (IUPHAR⁶²) (Fig.4B). In total, we tested 75 GPCRs with a cognate ONE-GO biosensor, and obtained concentration-dependent response curves for >95% of them (72 out of 75 receptors) (Fig.4B, [SI2]). Overall, there was a good correlation between EC_{50} values determined in our assays with ONE-GO biosensors and IUPHAR's data (Fig.4B, [SI3]). Although comparing our EC_{50} 's to the parameters from IUPHAR is limited by the contribution of differences in experimental conditions, these results indicate that detection of GPCR activation by ONE-GO biosensors is broadly reliable and sensitive.

We next asked if ONE-GO biosensors could distinguish the profiles of activation of multiple G proteins by the same GPCR when stimulated by more than one ligand. For this, we explored the activation of six different G proteins of the $G_{i/o}$ family upon stimulation of the μ -opioid receptor (MOR) by six different neuropeptides. Concentration dependence curves were generated for the 36 combination of ligand/ G protein, which revealed an overall pattern of higher potencies on G_{α_oA} , G_{α_oB} , and G_{α_z} compared to $G_{\alpha_{i1}}$, $G_{\alpha_{i2}}$, and $G_{\alpha_{i3}}$ for all ligands, and that Dynorphin A and β -endorphin had lower potency than the rest of the neuropeptides regardless of the G protein investigated (Fig.4C, [SI4]). These observations agree with recent evidence using an alternative biosensor system⁶³. We also expanded the characterization of ONE-GO biosensors by applying them to adhesion GPCRs, an understudied class of GPCRs with a unique mechanism of activation^{64,65}. For this, we used an engineered version of ADGRL3 (Latrophilin 3) that can be rapidly activated by thrombin by virtue of a fusion to a fragment of the PAR1⁶⁶. By blocking endogenous thrombin receptors in HEK293T cells with vorapaxar, we could determine that ADGRL3 activates G proteins of the $G_{12/13}$ family, but not of any of the other 3 G protein families (G_s , $G_{i/o}$, $G_{q/11}$) using ONE-GO biosensors (Fig.4D). Taken together, these results indicate that

ONE-GO biosensors can detect G protein activation by many GPCRs upon stimulation by diverse ligands and mechanisms.

Given the success with $G\alpha$ -GTP ONE-GO biosensors, we also generated ONE-GO biosensors for the other active signaling species generated upon GPCR stimulation, free $G\beta\gamma$ (Fig.S3C). For this, we leveraged the individual components of a previously described $G\beta\gamma$ BRET biosensor^{26,27}, which were assembled into a single plasmid (Fig.S3C). As a proof of principle, we showed that this design detected responses upon stimulation of G_s or G_{i3} heterotrimers by a cognate GPCR (Fig.S3C).

In summary, our results demonstrate that the ONE-GO biosensor design is broadly applicable to detect GPCR-mediated activation across and within G protein families with high sensitivity.

Parallel profiling of atypical antipsychotics across a large set of receptors

Given the advantages of delivery as a single genetic cargo and improved dynamic range of ONE-GO biosensors, we set out to demonstrate their suitability for applications in two broad areas: (1) scaling up throughput in experiments with cell lines, and (2) allowing the interrogation of endogenous GPCR activity, even in difficult to transfect primary cells. To start addressing the implementation of ONE-GO biosensors in the first area, we focused our attention on three widely prescribed atypical antipsychotics: brexpiprazole, iloperidone, and cariprazine⁶⁷. Although the exact mechanism of action of these drugs is unknown, the therapeutic benefit of atypical antipsychotics arises from their GPCR polypharmacology, i.e., engaging simultaneously multiple GPCRs, typically with partial agonism/antagonism. Nevertheless, the complex pharmacological profiles of atypical antipsychotics are also believed to underlie the wide range of side effects that vary from one specific drug to another. Although brexpiprazole⁶⁸, iloperidone^{69,70}, and cariprazine⁷¹ have been systematically characterized for binding to large panels of GPCRs, their functional effect as agonists and antagonists has been less systematic, mixing assay readouts and cell lines. We leveraged ONE-GO biosensors to expand and unify the functional characterization of the effect of these three drugs against a panel of 45 GPCRs regulated by neurotransmitters (Fig.5A, [SI5]). Each drug was assayed in the absence or presence of a canonical agonist for each GPCR to test agonist or antagonist activity, respectively. For each drug, all receptors were interrogated in parallel for a total of >500 simultaneous measurements (Fig.5A). As expected, the three drugs presented agonist and antagonist activities on numerous GPCRs. From a broad perspective, we found that brexpiprazole and cariprazine had similar profiles of agonism and antagonism, whereas iloperidone differed, as supported by principal component analysis (PCA) (Fig.5A). In broad strokes, iloperidone acts on similar targets as brexpiprazole and cariprazine, prominently serotonin, dopamine, and adrenergic receptors, but with more potent antagonist activity and poor agonist efficacy. At a more granular level, we recapitulated known features of these drugs, including their action on 5-HT1a, 5-HT2a or D2-like dopamine receptors^{68–71}, but also unknown ones. For example, we identified inverse agonism for iloperidone on 5-HT1b or 5-HT1d, or the full agonist activity of brexpiprazole and cariprazine on 5-HT1b and 5-HT2c, or of cariprazine on 5-HT1d (Fig.5A). All three drugs also antagonized the α_2C -AR. Overall, these findings demonstrate that the ONE-GO

biosensor platform can be leveraged to rapidly illuminate GPCR pharmacological profiles through functional interrogation of hundreds of conditions in parallel.

Pharmacogenomics of atypical antipsychotics

Next, we expanded the characterization of atypical antipsychotics by assessing how naturally-occurring variants in GPCR sequences influence drug action, a phenomenon known as pharmacogenomics. Pharmacogenomics of GPCR drug targets might account for many adverse side effects or varying drug efficacy across patient populations, but progress in improving prescription precision or clinical trial design has been hampered by the lack of approaches to systematically interrogate the functional consequences of GPCR variants^{72–74}. We leveraged ONE-GO biosensors to compare the pharmacological profiles of brexipiprazole, iloperidone, and cariprazine on GPCR variants bearing frequent single nucleotide polymorphisms (SNPs). We first carried out coarse-grained concentration-dependence studies to rapidly identify potential differences by assessing hundreds of experimental conditions in parallel (Fig.S5A, [SI6]). Although we recognized that these SNPs could affect total or surface expression of the GPCRs, our goal was to determine if the variant led to different functional outcomes regardless of the underlying cause. Most of the variants led to little or no differences (Fig.S5A), whereas four of them were selected for more granular concentration-dependence studies to define the putative differences: 5-HT1a A50V, 5-HT1a R220L, 5-HT1b F124C, 5-HT1b R230H (Fig.5B). While the A50V variant had no effect on the efficacy of serotonin, brexipiprazole or iloperidone on 5-HT1a, it made cariprazine a more efficacious antagonist, while reducing its agonist activity (Fig.5B). In contrast, 5-HT1a R220L reduced the efficacy of serotonin, but had no effect on any of the three antipsychotics (Fig.5B). As for, 5-HT1b variants, both F124C and R230H reduced the efficacy of iloperidone as an inverse agonist without changing the effects of brexipiprazole or cariprazine (Fig.5B). R230H but not F124C increased the efficacy of serotonin on 5-HT1b. Overall, these results reveal distinct functional interactions between widely prescribed antipsychotics and naturally-occurring GPCR genetic variants.

Profiling of G protein selectivity across short-chain fatty acid receptors

Many GPCRs, including those activated by short-chain fatty acids (SCFA), can promiscuously activate different classes of G proteins modulating distinct downstream pathways^{75,76}. Extracellular SCFA generated by the gut microbiota or as byproducts of eukaryotic metabolism regulate numerous physiological functions via GPCR activation^{77–79}. GPCR responses triggered by SCFA are shaped by the intersection of multiple variables, yet the relationship between these variables and the responses has not been systematically deconvoluted using a unified readout^{80,81}. The variables consist of multiple ligands [variable 1] that act on multiple GPCRs [variable 2] to promiscuously activate G proteins of different families [variable 3] (Fig.5C, Fig.S5B, [SI7]). We set out to profile these variables in parallel with ONE-GO biosensors for representative members from each of the 4 G protein families (G α s, G α i1, G α q, G α 13), as well as for different members of the G α _{i/o} family (G α i1, G α i3, G α oA). None of the receptor-ligand combinations led to a response with G α s ONE-GO, whereas G α q activation was only detected for FFAR2 and G α 13 activation was only detected with FFAR3 (Fig.5C, Fig.S5B). Both FFAR2-G α q and FFAR3-G α 13 responses were similar regardless of the SCFA ligand used. While all three receptors elicited activation

of $G_{i/o}$ proteins, they did so with different profiles. First, FFAR2 was a poor activator of G_{α_A} compared to FFAR3 and HCAR2. The SCFA ligand used also led to different responses across receptors. For example, efficacy and potency of $G_{i/o}$ responses decreased with the chain length of the SCFA for FFAR2, whereas HCAR2 showed the opposite pattern (Fig.5C, Fig.S5B). For the $G_{i/o}$ responses with FFAR3, the efficacy was similar for the three ligands, but potency was increased for ligands with longer chains (Fig.5C, Fig.S5B). In summary, these results shed light onto the complex G protein responses elicited by SCFA depending on the combination of available receptors and relative abundance of ligands.

Time-resolved parallel interrogation of GPCR responses

Next, we tested the suitability of ONE-GO biosensors to record multiple kinetic responses to GPCR ligands in parallel, instead of the end-point measurements described above. Time-resolved measurements can be useful not only to evaluate changes in activation/deactivation kinetics, but also to probe multiple modes of pharmacology like agonism, antagonism, and allosteric modulation from a single well^{82,83}. We measured time-resolved responses simultaneously across 48 wells with two different receptors (α_2A -AR and $GABA_B$ R), obtaining high reproducibility scores ($Z' > 0.9$), and resolving an expected delay in G protein deactivation kinetics after disabling RGS GAP mediated regulation with mutation G183S in $G_{\alpha i3}$ ^{84,85} (Fig.S5C).

Detection of endogenous GPCR activity in cells lines

To start investigating the detection of responses elicited by endogenous GPCRs, we generated stable HeLa cells expressing $G_{\alpha i3}$ ONE-GO or $G_{\alpha i3}/G\beta\gamma$ ONE-GO biosensors by lentiviral transduction followed by fluorescence-activated cell sorting (Fig.S6A–B). We previously showed that endogenous α_2 -adrenergic receptors in these cells led to detectable $G_{\alpha i}$ -GTP or free $G\beta\gamma$ responses with a different biosensor design named BERKY³⁰. We found that stimulation with an α_2 -adrenergic agonist gave rise to robust concentration-dependent responses in cells expressing different levels of $G_{\alpha i3}$ ONE-GO or $G_{\alpha i3}/G\beta\gamma$ ONE-GO biosensors, and that these responses were much larger than those observed previously in the same cells with $G_{\alpha i^*}$ -BERKY3 or $G\beta\gamma$ -BERKY3 biosensors, respectively³⁰ (Fig.S6A–B). Similarly, we compared side by side responses triggered by endogenously expressed opioid receptors in SH-SY5Y using $G_{\alpha i3}$ ONE-GO or $G_{\alpha i^*}$ -BERKY3 biosensor³⁰ (Fig.S6C), finding found that the ONE-GO biosensor outperformed the BERKY biosensor by eliciting a response almost 10-fold larger. Overall, these results demonstrate that ONE-GO biosensors detect responses triggered by different endogenous GPCRs in two separate cell lines, and that these responses largely exceed those measured by previously described biosensors that also detect endogenous GPCR responses.

Endogenous GPCR activity across a wide palette of primary cells

Next, we evaluated ONE-GO biosensors to detect endogenous GPCR responses in primary cells, which should better recapitulate physiologically-relevant conditions compared to immortalized or transformed cell lines. First, we succeeded in detecting kinetic and concentration-dependent responses triggered by endogenous adenosine or protease activated receptors in primary human cardiac fibroblasts transduced with lentiviruses of ONE-GO biosensors for G proteins representative for each one of the four families (Fig.6). We

expanded our studies to five other primary cell types representing different organs from both human and mouse origin: human bronchial smooth muscle cells, human umbilical vein endothelial cells, mouse lung fibroblasts, mouse astroglial cells, and mouse cortical neurons. To achieve neuron-specific expression, the CMV promoter was replaced by the synapsin I promoter in the lentiviral constructs. Figure 6 shows examples of responses detected in all these cell types, which account for all G protein families and at least 10 general types of receptors for diverse ligands. Figure S7 illustrates additional examples of ligand-biosensor pairs, including G_{α_A} responses, and controls assessing the specificity of the responses observed. For example, $G_{\alpha_i/o}$ responses were blocked by pertussis toxin⁸⁶, and G_{α_q} responses by YM-254890⁸⁷, whereas none of these treatments affected $G_{\alpha_{13}}$ responses, as expected (Fig.S7). Given the lack of inhibitors for G_{α_s} , the specificity of the responses was determined with selective antagonists for the presumed G_s -coupled GPCRs (Fig.S7). We also assessed potential differences in neurotransmitter responses depending on the subtype of neurons investigated (Fig.S7), finding that striatal neurons, compared to cortical neurons, gave larger responses to adenosine or the α_2 -adrenergic agonist brimonidine, whereas serotonin responses were smaller and baclofen and dopamine remained similar (Fig.S7). These results demonstrate the suitability and broad applicability of ONE-GO biosensors for the detection of endogenous GPCR responses mediated by any general type of G protein across an ample palette of distinct primary cell types.

Cell type specific G protein selectivity profiles of protease-activated receptor 1

A poorly understood but critical aspect of GPCR regulation is its context dependence. In other words, how does GPCR behavior depend on the cell type or state in which it is expressed? In the next sections, we provide three examples of how measuring endogenous GPCR activity reveals context-specific behavior; from how a receptor changes its profile of activation of different G protein families depending on cell type, to discrimination of G protein subtypes within the same family for some receptors in neurons, to remodeling of G protein activation profiles upon induction of disease-mimicking cell transformation.

Protease activated receptor 1 (PAR1) is known to promiscuously couple to G proteins of the $G_{i/o}$, $G_{q/11}$ and $G_{12/13}$ families^{75,76}. Although G protein coupling specificity has been systematically addressed with unified readouts in cell lines expressing exogenous PAR1⁷⁵, this has not been done for endogenous PAR1. We stimulated endothelial cells, lung fibroblasts, cardiac fibroblasts and astroglial cells with a PAR1-specific agonist and measured activation of G_{α_q} , G_{α_i3} , or $G_{\alpha_{13}}$ with ONE-GO biosensors (Fig.7A). All cell types exhibited a robust G_{α_q} response, but the relative strength of the G_{α_i3} and/or $G_{\alpha_{13}}$ responses varied across cell types giving rise to distinct profiles of G protein activation (Fig.7A, *left*). For example, G_{α_i3} responses were very weak or absent in endothelial cells and cardiac fibroblasts, whereas $G_{\alpha_{13}}$ responses were strong in cardiac fibroblast but only modest in the other three cell types (Fig.7A). The different response profiles could not be explained by differences in relative expression of the three biosensors, as determined by luminescence measurements (Fig.7A, *right*). These results reveal that the profile of PAR1 coupling to G proteins of different families depends on the cell type in which the receptor is expressed.

Some neuroinhibitory GPCRs discriminate across $G_{i/o}$ isoforms in primary neurons

A widely held tenet based on studies of GPCRs exogenously expressed in cell lines is that those that couple to $G_{i/o}$ proteins do not discriminate between members of the same family like $G_{\alpha i}$ and $G_{\alpha o}$ isoforms⁷⁵. We investigated $G_{\alpha i1}$ and $G_{\alpha oA}$ activation using ONE-GO biosensors in mouse cortical neurons upon stimulation with full agonists for $G_{i/o}$ coupled GPCRs, like $\alpha 2$ -adrenergic, 5-HT₁ serotonin, D₂-like dopamine, and A₁ adenosine receptors. While we detected robust $G_{\alpha i1}$ responses in all conditions, $G_{\alpha oA}$ responses were detected only upon stimulation with brimonidine ($\alpha 2$ -adrenergic agonist) and dopamine (Fig.7B). The lack of $G_{\alpha oA}$ response for serotonin and adenosine could not be attributed to poor health of the cell preparations because subsequent stimulation with dopamine in the same recordings led to responses (Fig.7B). It is also unlikely that the absence of response is due to lack of sensitivity, given that $G_{\alpha i1}$ responses with serotonin and adenosine are even larger than with brimonidine and dopamine (Fig.7B), so one would have expected proportionally larger $G_{\alpha oA}$ responses with the former pair of receptors compared to the latter. We confirmed that stimulation of A₁ adenosine receptors exogenously expressed in HEK293T cells led to activation of both $G_{\alpha i1}$ and $G_{\alpha oA}$, and that the relative activation of $G_{\alpha oA}$ compared to $G_{\alpha i1}$ is equivalent to that observed upon activation of the D₂ dopamine receptor (Fig.7B, *right box*), which contrasts with the observations in neurons. We conclude that some endogenous $G_{i/o}$ coupled GPCRs in primary cortical neurons can discriminate between $G_{\alpha i}$ and $G_{\alpha o}$ isoforms.

Myofibroblast transformation remodels the G protein selectivity profile of protease-activated receptor 1

To characterize changes in signaling behavior of endogenous GPCRs in the context of disease, we focused on PAR1 responses in a model of fibrotic transformation of cardiac fibroblasts. Conversion cardiac fibroblasts into myofibroblasts is a hallmark of heart failure upon fibrotic transformation^{88,89}. Although PAR1 receptors contribute to fibrotic transformation in the heart and other tissues^{90–94}, whether changes in their signaling behavior accompany the process of transformation and the specific features of these changes are unknown. To address this question, we measured activation of $G_{\alpha q}$, $G_{\alpha i3}$ and $G_{\alpha 13}$ with ONE-GO biosensors upon stimulation with a PAR1-specific agonist in a widely used model of fibrotic transformation (Fig.7C). This model consists of inducing the conversion of quiescent cardiac fibroblasts into myofibroblasts by TGF β stimulation^{95,96}, which we confirmed in our hands by detecting the upregulated expression of the fibrosis markers (Fig.7C, *left box*). Compared to quiescent cardiac fibroblasts, transformed myofibroblasts displayed a modest increase in $G_{\alpha q}$ activation, a marked increase in $G_{\alpha i3}$ activation, and no difference in $G_{\alpha 13}$ activation (Fig.7C). These differences could not be attributed to differences in expression of the components of the biosensors or the PAR1 receptor, as assessed by immunoblotting and/or measurements of luminescence (Fig.7C). The lack of difference in basal BRET ratios also indicates that fibrotic transformation does not alter G protein activity under unstimulated conditions (Fig.7C). Overall, these results not only indicate that PAR1 signaling is altered upon fibrotic transformation, but that the changes consist of a remodeling of the relative strength of activation of different G protein types, rather than a global upregulation or downregulation of the receptor responses.

DISCUSSION

The main advances provided by this work are both technical and conceptual. We generated a set of tools to interrogate GPCR activity with high fidelity and ease even when the receptors are expressed endogenously in their native cellular environment and leveraged these tools to provide insights into GPCR biology and pharmacology. The virtually universal applicability of ONE-GO biosensors is supported not only by their demonstrated suitability for a broad set of G proteins and receptors, but also by their implementation across a wide range of cell types (primary cultures and immortalized lines), scalable assay formats (endpoint or kinetic), and gene delivery approaches (transient transfection/ transduction or stable genomic integration). An important concept put forth by our findings with ONE-GO biosensors is that GPCR signaling is hardwired in a context-dependent manner, e.g. in specific cell types, even at the level of the signal transduction event most proximal to ligand-mediated receptor activation (e.g., loading of GTP on G proteins). The phenomenon of context-dependent signaling hardwiring, also known as *system bias*²⁰, has been a concern in GPCR drug discovery because it may underlie the lack of consistent translation of findings *in vitro* into the desired pharmacological properties *in vivo*^{13,20}. For example, ligand-induced signaling bias currently identified for a given drug candidate *in vitro* using mainstay approaches with transfected cell lines might become negated by system bias when the receptor is expressed endogenously in the target tissue. This issue could be alleviated by implementing ONE-GO biosensors to test drug candidates *in vitro* in cellular systems more relevant to the intended final application, like a primary cell type endogenously expressing the GPCR of interest. A similar approach could also open an avenue for personalized medicine by investigating endogenous GPCR activity in cells differentiated from induced pluripotent stem cells of patients. Other conceptual advances reported here by the implementation of the biosensors include the elucidation of structure-function relationships in G protein activation, and the discovery of unknown features of the pharmacological or pharmacogenomic profiles of widely prescribed drugs (antipsychotics) or of natural ligands (SCFA).

While our findings of context-dependent signaling reveal that system bias is a prevalent feature of endogenous GPCRs, we can only speculate about the possible causes. For example, although there could be differences in expression of PAR1 across the 4 cell types in which it was investigated, this is unlikely to cause the differences observed because these are changes in the *relative strength* of activation of different G proteins. The possible influence of GPCR and G protein stoichiometry was more definitely ruled out in the experiments testing PAR1 G protein activation remodeling upon fibrotic transformation, in which we confirmed no changes in the abundance of the different signaling components. Similarly, changes in receptor and/or G protein levels cannot explain our observations in neurons. We envision at least three general factors that could shape GPCR responses in a context-dependent manner. One is post-translational modifications. For example, it has been reported that PAR1 glycosylation differentially affects signaling by distinct G proteins⁹⁷. Another factor is compartmentalization. For example, GPCRs distributed to specific subcellular structures (e.g., synapses in neurons) or signaling complexes might have different accessibility to different G proteins^{25,98–101}. A third factor is the presence of molecules that directly affect the activity of GPCRs or G proteins, which can differ

across cell types and/or states. For example, GPCRs can be modulated by lipids like cholesterol or phosphatidylinositol 4,5-bisphosphate^{102–105}, or by proteins like Receptor Activity-Modifying Proteins (RAMPs)^{106–108}, and G proteins can be regulated by GAPs¹⁰⁹, GDIs¹¹⁰, and GEFs^{111–114}, among others^{115,116}.

An advantage of ONE-GO biosensors is that they only require the expression of low amounts of exogenous G α subunits, which bind to endogenous G $\beta\gamma$ to assemble functional G protein complexes that can be activated by GPCRs. On one hand, this prevents the loss of fidelity imposed by the use of specific combinations of exogenous G β and G γ subunits, which alters the signaling profiles of GPCRs^{29,117}. On the other hand, this minimizes the potential impact of gross overexpression on the signaling system under investigation, as demonstrated by the lack of measurable effects of ONE-GO biosensors on downstream signaling to modulate cAMP or kinases.

Limitations of the study

It is still a limitation over other biosensor systems like BERKY³⁰ that an exogenous G α needs to be expressed. It is therefore important for investigators to tailor the use of a given biosensor platform to their question of interest. In cases in which fidelity of the response is of utmost importance, BERKY biosensors are more suitable due to the complete absence of exogenous G proteins. However, there might be instances in which the larger dynamic range of ONE-GO biosensors compared to BERKY might justify a minimal compromise of fidelity due to expression of low amounts of G α . Another limitation to keep in mind for ONE-GO biosensors is that the tagging of G α could influence their behavior, although this is unlikely based on previous observations^{25,30,60}. A general concern for any biosensor is that it is theoretically impossible to record an activity with them without affecting the signaling process in *absolute terms*. For example, probes used for Ca²⁺ imaging can also chelate the divalent cation^{118,119} or EKAR-type kinase activity biosensors can compete with the native kinase substrates¹²⁰. What is critical to determine is whether the effect on the signaling process under investigation is insignificant in *practical terms*, as our results with ONE-GO biosensors confirm. Finally, it could be a limitation that there are no ONE-GO biosensors for some G α subunits.

STAR Methods

RESOURCE AVAILABILITY

Lead Contact—Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Mikel Garcia-Marcos (mgm1@bu.edu).

Materials Availability—All biosensor plasmids generated in this paper have been deposited to Addgene, including a kit for the 10 ONE-GO sensors under the CMV promoter (Addgene Kit #1000000224).

Data and Code Availability

- Gel and membrane original scans from all Figures were deposited on Mendeley at <http://dx.doi.org/10.17632/9tjcy8rg4t.1>.

- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Cell lines—HEK293T cells (ATCC cat# CRL-3216,) and HeLa cells (ATCC cat# CCL-2) were grown at 37°C, 5% CO₂ in DMEM (Gibco cat# 11965–092) supplemented with 10% FCS (HyClone cat# SH30073.03), 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine. SH-SY5Y cells (ATCC cat# CRL-2266) were grown at 37°C, 5% CO₂ in DMEM supplemented with 15% heat-inactivated FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine. SH-SY5Y stably expressing Gai*-BERKY3³⁰ were grown in the same medium as naïve cells supplemented with 100 µg/ml hygromycin.

Institutional approval for mouse experiments—All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Boston University Chobanian & Avedisian School of Medicine (PROTO202000018). C57BL/6N wild-type mice were from an in-house colony originally established with animals obtained from the Mutant Mouse Resource & Research Centers (MMRRC) at UC Davis.

Mouse primary cortical & striatal neuron culture—Neurons were isolated from neonatal mouse brains as previously described¹²¹ with modifications. Newborn mouse pups (P0) were euthanized by decapitation. Brains removed from the skull and placed in cold HBSS. The cerebrum was detached from other brain regions under a stereomicroscope by removal of the olfactory bulb and cerebellum, and meninges were peeled off with a tweezer. For cortical neurons, the cortex was dissected out with forceps by removing the hippocampus and the entire midbrain region. For striatal neurons, the hippocampus and the cortex were removed using forceps. The cortex or striatum was minced into approximately 1–2 mm pieces using a sterile razor blade, and digested with 0.05% (w:v) trypsin in HBSS for 10 min at 37°C. Trypsinized tissue was washed three times with HBSS to remove trypsin, and resuspended in DMEM supplemented with 10% FBS (Gibco cat# 2614–079), 100 U/ml penicillin, 100 µg/ml streptomycin (complete neuro DMEM) before passing through a sterile 40 µm cell strainer (Fisherbrand, cat# 22363547) to obtain a cell suspension. Approximately 75,000–100,000 cells were plated on 5 mm diameter coverslips (Word Precision Instruments cat# 502040; coated overnight with 0.1 mg/mL poly-L-lysine hydrobromide [Millipore Sigma cat# P9155], and washed 3x with HBSS) and placed in 96-well plates containing 200 µl of complete neuro DMEM for 4 h, before replacing one half of the volume of medium in each well with Neurobasal medium (GIBCO, cat# 21103049) with B-27 supplement (GIBCO, cat# 17504001) and 1x Glutamax-I (GIBCO, cat# 35050061) (complete neural medium). Approximately 48 h later (day in vitro 3, DIV3), half of the volume of medium in each well was replaced with complete neural media supplemented with 5 µM AraC to achieve a final concentration of 2.5 µM in the well. Beginning DIV5, half of the volume of medium in each well was replaced by fresh complete neural medium every other day.

Mouse primary cortical astroglial cell culture—Astrocyte-rich glial cultures were prepared from the cortex of neonatal mice using a protocol similar to that used for cortical neuron cultures except for the following differences. The initial tissue was obtained from neonatal mouse pups (P0–3)¹²¹, and after straining trypsin-digested tissues, the cell suspension was used to seed 1.5 million cells in each well of a poly-L-lysine coated 6-well plate in complete neuro DMEM. Media was changed the following day, and cells were subsequently split at a 1:2 ratio every 2–3 days by trypsinization followed by centrifugation at $180 \times g$ for 5 minutes before resuspending and reseeding in complete neuro DMEM. Cells were cultured for not more than 5 passages.

Mouse primary lung fibroblast cell culture—Lung fibroblasts were isolated from 8–16 week old mice as previously described¹²² with modifications. Mice were euthanized by CO₂ inhalation followed by cervical dislocation. An incision was made with scissors to open the peritoneal cavity and the skin was removed to expose the cardiothoracic cavity. The sternum was cut open and the ribcage was held open using hemostat forceps. An incision was made in the liver, and DPBS containing calcium and magnesium (Corning, cat# 21–030-CV) was slowly injected into the right ventricle of the heart using a 25-gauge needle to perfuse the lungs. Both lungs and the heart were then dissected out and placed in cold DPBS with calcium and magnesium. The heart and any remaining parts of the trachea or other connective tissue were removed, and the outside of the lungs was rinsed three times in DPBS containing calcium and magnesium. Lungs from 4 mice were minced into ~1 mm pieces using curved scissors and digested with 1 mg/ml collagenase type 1 (Worthington, cat# LS004194) and 1X dispase (Corning, cat# 354235) in 12 ml of DPBS containing calcium and magnesium for 45 min on a shaker at 37°C. The digested tissue was passed through a 40 μ m cell strainer (Fisherbrand, cat# 22363547) to obtain a cell suspension. Twenty ml of DMEM supplemented with 15% FBS, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 2 mM L-glutamine (complete fibroblast DMEM) were added to neutralize the digestion cocktail and the tube was spun at $300 \times g$ for 5 min. The pellet was resuspended in 20 ml of complete fibroblast DMEM and cells corresponding to tissue from 4 mice were seeded on two 10 cm tissue culture plates. Cells were washed three times with PBS the following day and fresh complete fibroblast DMEM was added. Two days later, cells were split at a 1:3 ratio by trypsinization followed by centrifugation at $250 \times g$ for 4 minutes before resuspending and reseeding in complete fibroblast media on a total of six 10 cm tissue culture plates. Three days later, all cells were harvested by trypsinization and split in 7 1.2 ml cryogenic vials (Fisherbrand, cat# 1050025) in complete fibroblast media supplemented with 20% FBS and 10% DMSO, and frozen in liquid nitrogen as passage 1 cells for further use. For experiments, these passage 1 cells were quickly thawed in a 30°C water bath, and seeded in a 10 cm culture dish in 15 ml of complete fibroblast media. Media was replaced the day after and cells cultured by splitting as described above for not more than 4 passages.

Human primary cardiac fibroblasts—Human cardiac fibroblasts were obtained from ScienCell (cat# 6340) and cultured as previously described¹²³. After thawing quickly in a 30°C water bath, ~350 thousand cells were seeded in each well of a 6-well plate precoated overnight with 0.4% Matrigel (Corning, cat# 356231) in DMEM, and cultured in fibroblast growth medium 3 (Promocell, cat# C-23025) supplemented with 10% growth medium

supplement (Promocell, C-39345), 5 μ M SB431542 (Selleckchem, cat# S1067), 100 U/ml penicillin, 100 μ g/ml streptomycin, and 2 mM L-glutamine. Every two days, cells were split at a 1:2 ratio by incubation in Accutase (Sigma cat# A6964) at 37°C for 3 min to detach cells, followed by centrifugation at 200 $\times$ g for 3 min before resuspending and reseeding in fibroblast growth medium 3 supplemented with 10% growth medium supplement, 5 μ M SB431542, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 2 mM L-glutamine. Cells were typically used within 5 passages, and never cultured for more than 9 passages.

Human primary umbilical vein endothelial cells—Human umbilical vein endothelial cells (HUVECs) were acquired from Lonza (cat# CC-2519) and cultured in EBM Basal Medium (Lonza, cat# CC-3121) supplemented with the EGM Singlequots Supplement Pack (Lonza, cat# CC-4133) following the supplier's recommendations. After thawing quickly in a 30°C water bath, ~600 thousand cells were seeded on a 10 cm tissue culture dish. Cells were split at a 1:2 ratio every 2 days by trypsinization (Lonza cat# CC-5012), followed by centrifugation at 200 $\times$ g for 3 min before resuspending and reseeding in EBM Basal Medium supplemented with the EGM Singlequots Supplement Pack. Cells were cultured for not more than 8 passages.

Human primary bronchial smooth muscle cells—Human bronchial smooth muscle cells were obtained from Promocell (cat# C-12561) and cultured in SMC Basal medium 2 (Promocell, cat# C-22262) supplemented with Growth Medium 2 supplement Mix (Promocell, cat# 39267), 100 U/ml penicillin, 100 μ g/ml streptomycin, and 2 mM L-glutamine following the supplier's recommendations. After thawing quickly in a 30°C water bath, ~200 thousand cells were seeded in each well of a 6 well plate. Cells were split at a 1:2 ratio every 3 days by trypsinization (Promocell cat# C41210), followed by centrifugation at 220 $\times$ g for 3 min before resuspending and reseeding in SMC Basal medium 2 supplemented with Growth Medium 2 supplement Mix, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 2 mM L-glutamine. Cells were cultured for not more than 5 passages.

Method Details

Plasmids—To generate plasmids encoding putative G protein-binding peptides fused to GST (amino acid sequences presented in Fig. 1A), complementary primers encoding the peptides and overhanging ends complementary to EcoRI and HindIII were annealed and inserted between the EcoRI and HindIII site of the previously described pGEX-KG-KB1753 plasmid¹²⁴ to replace the existing KB1753 sequence.

Plasmids encoding KB1691-Nluc (pcDNA3.1-mas-KB1691-Nluc) and KB2123-Nluc (pcDNA3.1-mas-KB2123-Nluc) with a membrane anchoring sequence (mas) were generated by replacing the GRK3ct sequence of pcDNA3.1-mas-GRK3ct-NLuc²⁷ by digestion with HindIII/BamHI and subsequent insertion of the KB1691 sequence (SRELAWGISEWLEEWG) or the KB2123 sequence (SSSELADYFGWDGWPG) flanked with a Gly-Gly linker on each end. Plasmids encoding KB1753-Nluc (pcDNA3.1-mas-KB1753-Nluc), GRK2^{RH}-Nluc (pcDNA3.1-mas-GRK2^{RH}-Nluc), and PRG^{RH}-Nluc (pcDNA3.1-mas-PRG^{RH}-Nluc) have been described previously³⁰. The plasmid encoding KB1691-mKate2 (pcDNA3.1-mas-KB1691-mKate2) was generated by replacing the Nluc

sequence of pcDNA3.1-mas-KB1691-Nluc by digestion with BamHI/XhoI and subsequent insertion by Gibson assembly of the sequence encoding for mKate2. The Plasmid encoding KB1753-mKate2 (pcDNA3.1-mas-KB1753-mKate2) was generated by replacing the KB1691 sequence of pcDNA3.1-mas-KB1691-mKate2 by digestion with HindIII/BamHI and subsequent insertion by Gibson of the KB1753 sequence. Plasmids encoding human G α s-67V (pcDNA3.1-G α s-67V), G α s-99V (pcDNA3.1-G α s-99V), and G α s-154V (pcDNA3.1-G α s-154V) have previously been described³⁸. Plasmids encoding rat G α i3-YFP (pcDNA3.1-G α i3-YFP)¹²⁵, mouse G α q-V (pcDNA3.1-G α q-V)¹⁰¹, and human G α 13-YFP (pcDNA3.1-G α 13-YFP)³⁰, all of which are internally tagged with the fluorescent protein at the α b- α c loop of the G protein, have been previously described. The plasmids encoding G α s/G α i chimeras were generated using Gibson assembly by swapping either CT-s, α N β 1-s, or both to CT-i and α N β 1-i on human G α s-99V, and swapping either CT-i, α N β 1-i, or both to CT-s and α N β 1-s on rat G α i3-YFP. The plasmid encoding FLAG-Ric-8B (pcDNA3.1-FLAG-Ric-8B) was a gift from Dr. Kirill Martemyanov and has been described previously¹²⁶. Plasmids encoding untagged G β 1 and G γ 2 (pcDNA3.1-G β 1, pcDNA3.1-G γ 2) have been described previously²⁶. The plasmid encoding Glosensor 22F was acquired from Promega (cat# E2301). The plasmid encoding cytosolic KB1691-Nluc (pcDNA3.1-cyto-KB1691-Nluc) was generated by mutation of the glycine in position 2 of pcDNA3.1-mas-KB1691-Nluc to alanine, which removes the myristoylation site of the membrane anchoring sequence. The plasmid encoding for Venus fused to the membrane anchoring sequence of kRas (pVenus-C1-Venus-K-Ras) was a gift from Dr. Nevin Lambert⁴¹. The pSPAX2 and pMD2.G plasmids for lentiviral packaging were acquired from Addgene (#12260 and #12259).

To generate plasmids encoding ONE-GO biosensors of G α -GTP species under a CMV promoter, sequences of G α proteins internally tagged with a YFP at a loop of the all-helical domain along with sequences from an IRES and an Nluc-fused detector module were inserted between the BamHI and MluI sites of vector pLVX-IRES-Hyg by Gibson assembly. The species of the G proteins and amino acid (aa) position of YFP insertion were as follows: G α s (human, aa99), G α i1 (rat, aa121), G α i2 (rat, aa114), G α i3 (rat, aa114), G α oA (rat, aa119), G α oB (rat, aa119), G α z (rat, 119), G α q (mouse, aa124), G α 12 (human, aa140), G α 13 (human, aa131). The IRES used for the ONE-GO biosensors (denoted IRES* in Fig. S3B) has previously been described⁵⁷, which includes a spacer sequence before the post-IRES start codon and results in diminished expression relative to the coding sequence right downstream of the promoter^{57,58}. For G α s ONE-GO, a T2A-Ric-8B sequence was inserted after KB1691-Nluc (without a stop codon). To generate SV40-G α i3 ONE-GO and SV40-G α s ONE-GO constructs, a previously described plasmid (pLVX-mVenus-MYC-DAPLE, based on the pLVX-IRES-Hyg backbone)¹²⁷ was digested with ClaI and SpeI to remove the CMV promoter and insert an SV40 promoter in its place to create pLVX-SV40-mVenus-MYC-DAPLE. The mVenus-MYC-DAPLE cassette between the BamHI and MluI sites was replaced by the same components of the G α i3 ONE-GO and G α s ONE-GO constructs described above. All ONE-GO biosensor sequences are provided in Supplemental Item 1 [SI 1]. To generate plasmids encoding ONE-GO biosensors of G α -GTP species under the neuron-specific promoter of human Synapsin (hSyn), the CMV promoter was removed from the plasmids described above by digestion with ClaI and SpeI, followed

by insertion of the hSyn promoter followed by a Xho I site in the same sites by Gibson assembly. The sequence of these plasmids (pLVX-hSyn-Gai1-YFP-IRES*-KB1753-Nluc, pLVX-hSyn-GaoA-YFP-IRES*-KB1753-Nluc, and pLVX-hSyn-Gaq-V-IRES*-GRK2^{RH}-Nluc) are provided in Supplemental Item 1 [SI 1].

To generate constructs encoding Gβγ ONE-GO biosensors for different Ga subunits, we first generated a parental plasmid without the Ga component (pLVX-CMV-IRES^{WT}-GRK3ct-Nluc-T2A-Gβ1-T2A-Gγ2-V). For this, the vector pLVX-IRES-Hygro was digested with NotI and MluI, and sequences for IRES^{WT}, GRK3ct-Nluc, and T2A-Gβ1-T2A-Gγ2-V were inserted by Gibson assembly. In contrast to IRES*, IRES^{WT} does not contain a spacer sequence before the post-IRES start codon and results in comparable expression relative to the coding sequence right downstream of the promoter. To generate plasmids encoding Gas/Gβγ ONE-GO and Gai3/Gβγ ONE-GO biosensors, human Gas or rat Gai3 were inserted in the NotI site of the parental plasmid described above. In the case of Gas, a BamHI site was included right before the start codon. Full sequences of parental Gβγ ONE-GO, Gas/Gβγ ONE-GO and Gai3/Gβγ ONE-GO biosensor are provided in Supplemental Item 1 [SI 1].

Plasmids encoding GPCRs were obtained from various sources (as indicated Key Resources Table) or subcloned into pcDNA3.1 from the PRESTO-Tango GPCR kit (Addgene Kit #1000000068) as described next. Briefly, GPCR sequences were amplified using primers that preserved the N-terminal FLAG tag present in the Tango library but introduced a stop codon after the end of the sequence of the native GPCR (i.e., it did not include the V2-tail, TEV site and tTA transcription factor present in the Tango constructs). The amplicons were inserted between the NotI and Apa I sites of pcDNA3.1 by Gibson assembly. The FLAG tag was not included for the following mGluR-encoding plasmids generated from the PRESTO-Tango library: pcDNA3.1(+)-GRM1, pcDNA3.1(+)-GRM2, pcDNA3.1(+)-GRM5, pcDNA3.1(+)-GRM7, and pcDNA3.1(+)-GRM8.

All point mutations were generated using QuikChange II following the manufacturer's instructions (Agilent, 200523).

Pulldown assays—Putative G protein-binding peptides fused to GST were expressed in BL21(DE3) *E. coli* transformed with the corresponding plasmids by overnight induction at 23°C with 1 mM isopropyl β-D-1-thio-galactopyranoside (IPTG). IPTG was added when the OD600 reached ~0.8. Bacteria pelleted from 50 ml of culture were resuspended at 4°C in 10.5 ml of lysis buffer (50 mM NaH₂PO₄, pH 7.4, 300 mM NaCl, 10 mM imidazole supplemented with a protease inhibitor mixture of 1 μM leupeptin, 2.5 μM pepstatin, 0.2 μM aprotinin, and 1 mM phenylmethylsulfonyl fluoride). The cell suspension was supplemented with 1% (v/v) Triton X-100 and sonicated (3 pulses of 20 s separated by 30 s intervals for cooling). The resulting lysate was cleared by centrifugation at 4,300 × g for 40 min at 4°C. The supernatant for each construct was collected and 2 ml aliquots were frozen until use.

For pulldown assays, an aliquot of lysate containing ~25–50 μg of each GST-fused peptide was incubated with ~20 μl of glutathione-agarose beads (Thermo, 16100) at 4°C for 90 min. Beads were washed twice with lysis buffer and resuspended in 250 μl of binding

buffer (50 mM Tris-HCl, 100 mM NaCl, 0.4% NP-40, 10 mM MgCl₂, 5 mM EDTA, 1 mM DTT) supplemented with 30 μ M GDP, 30 μ M AlCl₃, and 10 mM NaF. His-tagged G α s or G α o, purified as described previously^{128,129}, was diluted in binding buffer supplemented with 30 μ M GDP, 30 μ M AlCl₃, and 10 mM NaF to obtain 2 μ g of protein in 50 μ l, and incubated at 30°C for 60 min. GDP-AlF₄⁻-loaded G proteins were centrifuged at 14,000 $\times$ g for 3 min before addition to tubes containing the GST-fused peptides immobilized on glutathione-agarose beads. Tubes were incubated for 4 h at 4°C with constant rotation. Beads were washed three times with 1 ml of wash buffer (4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, pH 7.4, 137 mM NaCl, 2.7 mM KCl, 0.1% (v/v) Tween 20, 10 mM MgCl₂, 5 mM EDTA, 1 mM DTT) supplemented with 30 μ M GDP, 30 μ M AlCl₃, and 10 mM NaF, and resin-bound proteins were eluted with Laemmli sample buffer by incubation at 37°C for 10 min. Proteins were separated by SDS-PAGE and immunoblotted with antibodies as indicated under “Protein Electrophoresis and Immunoblotting.”

Bioluminescence Resonance Energy Transfer (BRET) measurements in

HEK293T cells—HEK293T cells were seeded on 6-well plates (~400,000 cells/well) coated with 0.1% gelatin (Sigma-Aldrich cat# G1393), and transfected ~24 h later using the calcium phosphate method. For experiments with bi-molecular biosensors consisting of YFP-tagged G α proteins and Nluc-fused detector modules (Fig. 1, 2, 3; Fig. S1, S2, S3), cells were transfected in the combinations indicated in the figures with the following plasmids (amount per well indicated in parenthesis): G α s-67V (1 μ g), G α s-99V (1 μ g), G α s-154V (1 μ g), G α i3-YFP (1 μ g), G α q-V (1 μ g) or G α 13-YFP (1 μ g), KB1691-Nluc (0.05 μ g), KB2123-Nluc (0.05 μ g), KB1753-Nluc (0.05 μ g), GRK2^{RH}-Nluc (0.05 μ g), PRG^{RH}-Nluc (0.05 μ g), Ric-8B (0.1 μ g), G β 1 (0.2 μ g), G γ 2 (0.2 μ g), and different GPCRs (0.2 μ g). For the bystander G α s biosensor system (Fig. S1D), cells were transfected with the following plasmids (amount per well indicated in parenthesis): G α s (0.5 μ g), cytosolic KB1691-Nluc (0.01 μ g), Venus-kRas (0.25 μ g), and β 2AR (0.2 μ g). For experiments with Venus-tagged G β γ and Nluc-fused GRK3ct (Fig. S4A), cells were transfected with the following plasmids (amount per well indicated in parenthesis): G α s or G α i3 (1 or 0.5 μ g, respectively), G β 1-V (0.2 μ g), G γ 2-V (0.2 μ g), GRK3ct-Nluc (0.05 μ g). Unless otherwise indicated in the figures, the amount of plasmid transfected per well for G α -GTP ONE-GO biosensors and G β γ ONE-GO biosensors was 0.05 μ g and 1 μ g, respectively, and the amount of GPCR-encoding plasmids co-transfected was 200 ng, except for the plasmid PAR1-ADGRL3 in Fig. 4D which was 500ng to replicate previously described conditions, or plasmids encoding A1R, P2Y6 and P2Y12 in [Supplemental Item 2], which was 5 ng to reduce basal activity.

Six hours after transfection, media was replaced by fresh complete medium or medium with reduced serum (0.1% FCS) in the case of experiments with glutamate receptors (Fig. 4B; [Supplemental Item 2]). Cells were harvested 18–24 h later for luminescence measurements by washing with PBS once and gentle scraping in 1 ml of PBS. Cells were centrifuged for 5 min at 550 $\times$ g, and resuspended in BRET buffer (140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 0.37 mM NaH₂PO₄, 20 mM HEPES pH 7.4, 0.1% glucose) at a concentration of approximately 1 million cells/ml. Twenty-five to fifty thousand cells were added to a white opaque 96-well plate (Opti-Plate, PerkinElmer cat# 6005290) and mixed

with the nanoluciferase substrate Nano-Glo (Promega cat# N1120, final dilution 1:200) for 2 min before measuring luminescence in a POLARstar OMEGA plate reader (BMG Labtech) at 28°C. Luminescence was measured at 460 ± 40 nm and 535 ± 15 nm, and BRET signal was calculated as the ratio between the emission intensity at 535 nm divided by the emission intensity at 460 nm.

For kinetic BRET measurements, luminescence signals were measured every 0.24 s (with a signal integration time of 0.24 s) for the duration of the experiment. Reagents were added to the wells during live measurements using injectors. BRET data are presented as the difference from baseline BRET signal [Δ BRET (baseline)] by subtracting the average of signal pre-stimulation from all data points. A similar procedure with minor modifications was followed for time-resolved multi-well experiments (Fig. S5C). Briefly, cells were added to 48 wells of an assay plate and incubated with CTZ400a as the luciferase substrate (GoldBio cat# C-320-1; final concentration of 10 μ M) before measuring luminescence in each well every 15 seconds. The Z' values for the time-resolved multi-well experiments were calculated as described previously¹³⁰ by using the formula $Z' = 1 - [3 * (\delta_{\text{positive}} + \delta_{\text{negative}}) / |\mu_{\text{positive}} - \mu_{\text{negative}}|]$, where δ is the standard deviation, μ is the mean, and positive / negative indicates presence of absence of agonist, respectively.

For endpoint BRET measurements to determine concentration dependent responses, CTZ400a (final concentration of 10 μ M) was used as luciferase substrate, and the BRET signal (535 nm luminescence / 460 nm luminescence) was measured every minute for 5 minutes with a signal integration time of 0.32 s for each measurement. Calculations were done with data corresponding to BRET signals at different time points depending on the kinetics of the response for different receptors and G proteins. BRET data are presented as the difference from BRET signal relative to a condition without agonist [Δ BRET (no agonist)]. Where indicated, the EC_{50} and pEC_{50} values were determined by using a 3-parameter sigmoidal curve-fit in Prism (GraphPad). To compare EC_{50} values experimentally determined with ONE-GO biosensors for different GPCRs and existing curated pharmacological parameters (Fig. 4B), we extracted pK_d , pK_i or pE/IC_{50} values for from the IUPHAR database⁶², with the following prioritization based on availability : $pK_d > pK_i > pE/IC_{50}$. The data was extracted for GPCRs of the same species as the ones used in the experiments with ONE-GO sensors, and when a range of values were given the midpoint was used. For some experiments, the data was further processed in the following ways. For Fig. 2C, D the Δ BRET values for each G protein chimera (shown in Fig. S2) were normalized by using the maximal Δ BRET value obtained with the cognate wild-type G protein for each GPCR as 1. For Fig. 5A, the Δ BRET values were normalized by using the Δ BRET value obtained upon stimulation with an EC_{80} concentration of the cognate agonist for each GPCR as 1. Data processed this way was used to generate the Principal Component Analysis (PCA) presented in this panel using the PCA tool with the centered method and with all principal components selected in Prism (GraphPad). For Fig. S5A, the Δ BRET values were normalized by using the Δ BRET value obtained upon stimulation with an EC_{100} concentration of the cognate agonist for each wild-type GPCRs as 1. For Fig. 5C, the Δ BRET values were first corrected by subtraction of BRET values obtained with cells treated with the same ligands but without GPCR transfection (shown in Fig. S5B), followed by normalization using the corrected Δ BRET value corresponding to the maximal response

observed for a given G protein across all three GPCR as 1. For curves in Fig. S5B with detectable responses ($> 0.001 \Delta\text{BRET}$) that could not be reliably fit to accurately determine EC_{50} values, an arbitrary EC_{50} value of 30 mM was given.

At the end of some BRET experiments, a separate aliquot of the same pool of cells used for the luminescence measurements was centrifuged for 1 min at $14,000 \times g$ and pellets stored at -20°C for subsequent immunoblot analysis (see “Protein electrophoresis and Immunoblotting” section below).

Luminescence-based cAMP measurements in cells—Approximately 300,000 HEK293T cells were seeded on each well of 6-well plates coated with 0.1% (w/v) gelatin, and transfected with plasmids ~24 hr later using the calcium phosphate method. For experiments testing the effect of the multi-plasmid $\text{G}_{\alpha s}$ sensor system on cAMP signaling (Fig. 1D), cells were transfected with the following plasmids (amount per well indicated in parenthesis): $\text{G}_{\alpha s}$ -99V (1 μg), FLAG-Ric-8B (0.1 μg), $\text{G}\beta 1$ (0.2 μg), $\text{G}\gamma 2$ (0.2 μg), $\beta 2\text{AR}$ (0.2 μg), with or without KB1691-Nluc (0.05 μg) and supplemented with pcDNA3.1 to equalize total amount of DNA per well. For experiments testing the effect of G_{α} ONE-GO biosensors on cAMP signaling (Fig. 3D), cells were transfected with the following plasmids (amount per well indicated in parenthesis): $\text{G}_{\alpha s}$ or $\text{G}_{\alpha i 3}$ ONE-GO biosensor (0.05 μg), $\alpha 2_{\text{A}}\text{-AR}$ (0.2 μg ; only in combination with $\text{G}_{\alpha i 3}$ ONE-GO) and supplemented with pcDNA3.1 to equalize total amount of DNA per well. The amount of Glosensor 22F plasmid transfected per well for all experiments was 0.8 μg . Cell medium was changed 6 h after transfections.

Approximately 16–24 h after transfection, cells were washed and gently scraped in room temperature PBS, and centrifuged (5 min at $550 \times g$). For kinetic measurements, cells were resuspended in 750 μl Tyrode’s buffer (140 mM NaCl, 5 mM KCl, 1 mM MgCl_2 , 1 mM CaCl_2 , 0.37 mM NaH_2PO_4 , 24 mM NaHCO_3 , 10 mM HEPES and 0.1% glucose, pH 7.4). Two-hundred μl of cells were mixed with 200 μl of 5 mM D-luciferin K^+ salt (GoldBio, LUCK-100) diluted in Tyrode’s buffer and incubated at 28°C for 15 minutes. Ninety μl of cells pre-incubated with D-luciferin were added to a white opaque 96-well plate (Opti-Plate, PerkinElmer Life Sciences, 6005290) before measuring luminescence without filters at 28°C every 10 s in a BMG Labtech POLARStar Omega plate reader. Agonists were added as indicated in the figures during the recordings using built-in injectors. Kinetic traces are represented as raw luminescence signal.

For concentration dependence experiments, cells were washed and scraped as above, but resuspended in 300 μl Tyrode’s buffer. Two-hundred and forty μl of cells were mixed with 240 μl of 5 mM D-luciferin K^+ salt diluted in Tyrode’s buffer and incubated at 28°C for 15 minutes. For experiments testing the effect of $\text{G}_{\alpha s}$ ONE-GO expression, 20 μl of different concentrations of isoproterenol diluted in Tyrode’s buffer at 5X the final concentration desired in the assay were added to wells of a white opaque 96-well plate and further diluted with 57.6 μl of Tyrode’s buffer. Reactions were initiated by addition of 22.4 μl of the suspension of cells pre-incubated with D-luciferin and luminescence measurements were immediately started. Luminescence signal was measured without filters at 28°C every 30 s in a BMG Labtech POLARStar Omega plate reader. For each concentration of isoproterenol,

the response values were calculated by averaging the 3 time points around the peak of the kinetic trace (270, 300, and 330 s after start of measurement) and normalizing them by using the response at maximal concentration of isoproterenol as 100. For experiments testing the effect of G*ai*3 ONE-GO expression, 20 μ l of different amounts of brimonidine diluted in Tyrode's buffer at 4X the final concentration desired in the assay were added to wells of a white opaque 96-well plate, and further diluted by addition of 37.6 μ l of Tyrode's buffer. Reactions were initiated at room temperature by addition of 22.4 μ l of the cell suspension pre-incubated with D-luciferin, and 2 minutes later 20 μ l of 500 nM isoproterenol (to achieve a final concentration of 100 nM in the assay) diluted in Tyrode's buffer were added, followed immediately by luminescence measurements as was done above for G*as* ONE-GO. For each concentration of brimonidine, response values were calculated by averaging the 3 time points around the peak of the kinetic trace (270, 300, and 330 s after start of measurement) and normalizing them by using the response in the absence of brimonidine as 100.

At the end of some experiments, a separate aliquot of the same pool of cells used for the measurements was centrifuged for 1 min at $14,000 \times g$ and pellets stored at -20°C for subsequent immunoblot analysis (see "Protein electrophoresis and Immunoblotting" section below).

BRET measurements in HeLa cells—HeLa cells stably expressing G*ai*3 ONEGO or G*ai*3/G*βγ* ONEGO were generated by lentiviral transduction followed by Fluorescence-Activated Cell Sorting (FACS). Lenti-X 293T (Cat# 632180, Takara Bio) cells were seeded on 6-well plates ($\sim 400,000$ cells/well) coated with 0.1% gelatin and transfected the next day using the polyethylenimine (PEI) method¹³¹ at a 2:1 PEI:DNA ratio with the following plasmids (amount of DNA per well in parenthesis): pLVX-CMV-G*ai*3-YFP-IRES*-KB1753-Nluc or pLVX-CMV-G*ai*3-IRES^{WT}-GRK3ct-Nluc-T2A-G*β*1-T2A-G*γ*2-V (1.8 μ g), psPAX2 (1.2 μ g), and pMD2.G (0.75 μ g). The DNA was added to 200 μ l of DMEM and mixed with 7.5 μ l of PEI reagent (Polysciences Inc cat# 23966). Tubes were incubated at room temperature for 15 min before adding to cells. Six hours after transfection, media was replaced with DMEM supplemented with 10% FCS, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 2 mM L-glutamine (complete medium). Lentivirus-containing media was collected 24 hr and 48 hr after transfection, centrifuged at $1500 \times g$ for 5 min after pooling, and filtered through a 0.45- μ m surfactant-free cellulose acetate (SFCA) membrane filter (Corning cat# 431220).

HeLa cells were seeded on 6-well plates ($\sim 200,000$ cells per well) precoated with 0.1 mg/mL poly-L-lysine hydrobromide (overnight incubation followed by 3 washes with HBSS) and transduced the next day by a 48 hr incubation in 2 ml of a 1:1 mix of lentivirus-containing supernatants described above mixed with fresh complete media, supplemented with 6 μ g/ml of polybrene (Tocris Bioscience cat# 7711/10). Cells were first transferred from the 6-well plate to a 10-cm plate after virus transduction for expansion. Once the 10-cm plate was confluent, cells were further expanded into 15-cm dishes to have enough material for sorting as described next. For FACS, HeLa cells were trypsinized, resuspended in complete medium, and 7.5 million cells were transferred to a 15 ml conical tube. Cells were washed $3 \times$ with 10 ml cold PBS by cycles of centrifugation ($300 \times g$ for 3 minutes),

aspiration, and resuspension. Cells were resuspended in 1.5 ml cold PBS and stored on ice until sorting. A subset of the trypsinized HeLa cells were resuspended in complete DMEM containing DAPI (1 µg/ml), washed as described above, and used for the gating process. Cell sorting was performed on a Moflo Astrios EQ (Beckman Coulter), and the 488_{ex}/513_{em} nm fluorescence channel (Voltage: 354 nV) was used for positive selection. Cells with fluorescent intensity above 300 were collected as “*High sensor expression*”, and cells with fluorescent intensity from 80 to 300 were collected as “*Low sensor expression*”. Cells were collected and seeded in a 6-well plate with complete DMEM for expansion.

For BRET experiments, HeLa cells stably expressing Gai3 ONEGO or Gai3/Gβγ ONEGO were seeded on 5 mm glass coverslips precoated with 0.1 mg/mL poly-L-lysine hydrobromide (overnight incubation followed by 3 washes with HBSS) and placed in a 96-well plate (~35,000 cells per well). Recordings were done 24 h after seeding as described next. Coverslips were washed with 200 µl BRET buffer and transferred to a well of a white opaque 96-well plate containing BRET buffer and Nano-Glo (final dilution 1:200) with tweezers, followed by incubation in the dark for 2 min at room temperature before measuring luminescence in a POLARstar OMEGA plate reader (BMG Labtech). Measurements and data processing were done as described in “BRET measurements in HEK293T cells” except that measurements with Gai3/Gβγ ONEGO were made every 0.48 s (with a signal integration time of 0.48 s).

To collect samples for immunoblotting, HeLa cells were seeded on a 6-well plate as described above, scraped in PBS and spun at 550 × g for 5 min. Pellets were processed for immunoblotting as described below in “Protein electrophoresis and Immunoblotting”.

Production of concentrated lentiviral particles—Lentiviruses used for transduction of SH-SY5Y cells and primary cells were concentrated after large scale packaging as described previously^{131,132}. Lenti-X 293T cells were plated on 150 mm diameter dishes (~2.5 million cells / dish) and cultured at 37°C, 5% CO₂ in DMEM supplemented with 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine. After 16–24 h, cells were transfected using the polyethylenimine (PEI) method¹³¹ at a 2:1 PEI:DNA ratio with the following plasmids (amount of DNA per dish in parenthesis): psPAX2 (18 µg), pMD2.G (11.25 µg) and Ga ONE-GO biosensor (27 µg). Approximately 16 h after transfection, media was replaced. Lentivirus containing media was collected 24 and 48 h after the initial media change (~70 mL per dish and 4 dishes for each construct). Media was centrifuged for 5 min at 900 × g and filtered through a 0.45 µm sterile PES filter (Fisherbrand cat# FB12566505). Filtered media was centrifuged for ~18 h at 17,200 × g at 4°C (Sorvall RC6+, ThermoScientific F12–6×500 LEX rotor) to sediment lentiviral particles. Pellets were washed and gently resuspended in 1 mL of PBS and centrifuged at 50,000 × g for 1 h at 4°C (Beckman Optima MAX-E, TLA-55 rotor). Pellets were resuspended in 500 µl of PBS to obtain concentrated lentiviral stocks that were stored at –80°C in aliquots. Each aliquot was thawed only once and used for less than a week stored at 4°C for subsequent experiments.

BRET measurements in SH-SY5Y cells—Naïve SH-SY5Y cells and SH-SY5Y cells stably expressing Gai*-BERKY3³⁰ were seeded on 35 mm plates (~700,000 cells/plate)

coated with 0.1% gelatin. The following day, naïve cells were transduced by replacing the media with fresh medium supplemented with concentrated lentiviral particles of the Gai3 ONE-GO biosensor under the CMV promoter (1:400 final dilution). Approximately 18 h later, lentivirus-containing media was replaced with fresh medium. Both SH-SY5Y cells stably expressing Gai*-BERKY3 and cells expressing the Gai3 ONE-GO sensor were harvested for luminescence measurements approximately 48 h after plating.

Briefly, cells were washed with PBS once, scraped, and spun at $550 \times g$ for 5 min. Cells were resuspended in BRET buffer at a concentration of approximately 1 million cells/ml. For luminescence measurements, 40,000–100,000 cells were added to a white opaque 96-well plate and kinetic BRET measurements were performed as described in “Bioluminescence Resonance Energy Transfer (BRET) measurements in HEK293T cells”. At the end of some BRET experiments, a separate aliquot of the same pool of cells used for the luminescence measurements was centrifuged for 1 min at $14,000 \times g$ and pellets stored at -20°C for subsequent immunoblot analysis (see “Protein electrophoresis and Immunoblotting” section below).

BRET measurements in primary cell cultures—ONE-GO biosensors were expressed in primary cells by lentiviral transduction according to the specific procedures described below using concentrated stocks described in “Production of concentrated lentiviral particles”. All primary cells were transduced with lentiviral constructs expressing the ONE-GO biosensors under the CMV promoter, except for neurons, where ONE-GO biosensors were expressed under the human synapsin I (hSyn) promoter. Cell seeding, viral dilutions, and other parameters are detailed next for each one of the cell types investigated. Mouse lung fibroblasts were seeded on 5 mm glass coverslips precoated with 0.1% gelatin (5 min incubation at room temperature followed by aspiration) and placed in a 96-well plate (15,000 cells per well). Approximately 18 h after seeding, cells were transduced by replacing the media with 100 μl of fresh media supplemented with 6 $\mu\text{g/ml}$ polybrene and the following lentiviruses (final dilution in parenthesis): Gai1 ONE-GO (1:500), Gai3 ONE-GO (1:500), Gai13 ONE-GO (1:500). Plates were spun at $600 \times g$ for 30 min and returned to the incubator. Media was replaced ~ 24 h later. BRET recordings were done 48 or 72 h after the addition of virus as described below. Mouse astroglial cells were seeded on 5 mm glass coverslips precoated with 0.1 mg/mL poly-L-lysine hydrobromide (overnight incubation followed by 3 washes with HBSS) and placed in a 96-well plate (40,000 cells per well). Approximately 18 h after seeding, cells were transduced by replacing the media with 100 μl of fresh media supplemented with 6 $\mu\text{g/ml}$ polybrene and the following lentiviruses (final dilution in parenthesis): Gai1 ONE-GO (1:250), Gai3 ONE-GO (1:500), Gai13 ONE-GO (1:100–1:250). Plates were spun at $600 \times g$ for 30 min and returned to the incubator. Media was replaced ~ 24 h later. BRET recordings were done ~ 48 h post-transduction as described below. Mouse cortical and striatal neurons were seeded as explained above in “*Mouse primary cortical & striatal neuron culture*”. On DIV7, cells were transduced by replacing half of the media with fresh media containing the following lentiviruses (final dilution in parenthesis): Gai1 ONE-GO (1:200), Gai11 ONE-GO (1:200), Gai13 ONE-GO (1:200). Half of the media was replaced with fresh medium ~ 2 h later and BRET recordings were done on DIV12–19 for cortical neurons, or DIV18–20 for

striatal neurons, as described below. Human bronchial SMCs were seeded on 5 mm glass coverslips precoated with 0.1% gelatin and placed in a 96-well plate (15,000 cells per well). Approximately 18 h after seeding, cells were transduced by replacing the media with 100 μ l of fresh media supplemented with 6 μ g/ml polybrene and the following lentiviruses (final dilution in parenthesis): Ga α q ONE-GO (1:1,500), Ga α s ONE-GO (1:1,500), Ga α 13 ONE-GO (1:1,500). Plates were then spun at 600 $\times$ g for 30 min and returned to the incubator. Media was replaced ~24 h later, followed by BRET recordings that same day as described below. HUVECs were seeded on 5 mm glass coverslips precoated with 0.1% gelatin and placed in a 96-well plate (15,000 cells per well). Approximately 18 h after seeding, cells were transduced by replacing the media with 100 μ l of fresh media supplemented with 6 μ g/ml polybrene and the following lentiviruses (final dilution in parenthesis): Ga α q ONE-GO (1:2,000), Ga α s ONEGO (1:500), Ga α i3 ONE-GO (1:2,000), Ga α 13 ONE-GO (1:500). Plates were then spun at 600 $\times$ g for 30 min and returned to the incubator. Media was replaced ~24 h later, followed by BRET recordings the same day as described below. Human cardiac fibroblasts were seeded on 5 mm glass coverslips precoated overnight with 0.4% Matrigel in DMEM and placed in a 96-well plate (13,000 cells per well). Approximately 18 h after seeding, cells were transduced the following ways depending on experimental purposes. For Fig. 6 and Fig. S7, cells were washed twice with HBSS to remove SB431542. Then, cells were incubated in 100 μ l of fresh media supplemented with 6 μ g/ml polybrene and the following lentiviruses (final dilution in parenthesis): Ga α q ONE-GO (1:4,000–1:5,000), Ga α s ONE-GO (1:1,000), Ga α i3 ONE-GO (1:2,000 for adenosine stimulated cells, 1:4,000–1:5,000 for TRAP-6 stimulated cells), Ga α 13 ONE-GO (1:4,000–1:5,000). Plates were spun at 600 $\times$ g for 30 min, returned to the incubator and media was replaced ~24 h later, followed by BRET recordings 2 h later when testing the effect of adenosine or 24 h later when testing the effect of TRAP-6. For Fig. 7A, cells were kept in media supplemented with 5 μ M SB431542 throughout. Approximately 18 h after seeding, cells were incubated in 100 μ l of fresh media supplemented with 6 μ g/ml polybrene and the following lentiviruses (final dilution in parenthesis): Ga α q ONE-GO (1:2,000), Ga α i3 ONE-GO (1:2,000), Ga α 13 ONE-GO (1:2,000). Plates were spun at 600 $\times$ g for 30 min, returned to the incubator and media was replaced ~24 h later, followed by BRET recordings 24 h later. For Fig. 7C, cells were washed twice with HBSS to remove SB431542 approximately 18 h after seeding. Then, cells were incubated in 100 μ l of fresh media supplemented with 6 μ g/ml polybrene and the following lentiviruses in the presence or absence of TGF β (final dilutions without/with 1 nM TGF β respectively in parenthesis): Ga α q ONE-GO (1:2,000/1:5,000), Ga α i3 ONE-GO (1:2,000/1:5,000), Ga α 13 ONE-GO (1:2,000/1:5,000). Plates were spun at 600 $\times$ g for 30 min, returned to the incubator and media was replaced ~48 h later, followed by BRET recordings 2 h later.

Kinetic BRET recordings were done the same way for all cell types. Briefly, coverslips were washed with 200 μ l BRET buffer and transferred to a well of a white opaque 96-well plate containing BRET buffer and Nano-Glo (final dilution 1:200) with tweezers, followed by incubation in the dark at room temperature for 2 min before measuring luminescence in a POLARstar OMEGA plate reader (BMG Labtech). Measurements and data processing were done as described in “BRET measurements in HEK293T cells” except that measurements were taken every 0.96 s (with a signal integration time of 0.96 s). Where indicated in the

figures or figure legends, cells were pretreated with pertussis toxin (overnight), YM-254890 (2 min), or a GPCR antagonist (2 min). To determine “Max Δ BRET” (Fig. 7A, 7C), 9 data points centered around the maximum response were averaged. For some representations in Fig. 7, Max Δ BRET values were normalized by using the Max Δ BRET value obtained with Gαq ONE-GO for each cell type as 1. The “*Basal BRET*” and “*Luminescence*” values reported in Fig. 7C were obtained by averaging the first 10 data points of 535/460 ratios or direct counts in the 460 nm channel, respectively.

For endpoint BRET measurements to determine concentration-dependent responses with cardiac fibroblasts (Fig. 6), coverslips were washed and transferred to an assay plate as explained above. A 10X agonist solution was simultaneously added to all wells using a multi-channel pipette, and BRET signal (535 nm luminescence / 460 nm luminescence) was measured every minute for 5 minutes with a signal integration time of 0.96 s for each measurement. Calculations were done with data corresponding to BRET signals at different time points depending on the kinetics of the response for different receptors and G proteins. BRET data are presented as the difference from BRET signal relative to a condition without agonist [Δ BRET (no agonist)]. The Gαi3 ONE-GO concentration response curve was generated by doing kinetic BRET measurements as explained above and taking the difference in Δ BRET at max amplitude for each concentration compared to BRET buffer only injections.

To collect samples of human cardiac fibroblasts for immunoblotting, cells were seeded on a glass bottom 12-well plate precoated overnight with 0.4% Matrigel in DMEM (~140,000 cells per well). Transductions and TGFβ treatments were done exactly as described above for the BRET measurements. Cells were washed with PBS and 90 μl of ice-cold lysis buffer was added to each well before proceeding with the rest of the steps described in “Protein electrophoresis and Immunoblotting” section below.

RT-qPCR of cardiac fibroblast mRNA—Human cardiac fibroblasts were seeded on a glass-bottom 12-well plate precoated overnight with 0.4% Matrigel in DMEM (~140,000 cells per well). Approximately 18 h after seeding, cells were washed 2 × with HBSS, and then incubated in 1 ml of fresh media without SB431542 supplemented (or not) with 1 nM TGFβ. Approximately 48 h later, RNA was extracted using the PureLink RNA Mini kit (ThermoFisher cat# 12183018A) and stored at −80°C. Two-hundred and fifty ng of RNA were used for reverse transcription reactions using the LunaScript RT SuperMix (New England BioLabs cat# E3010). For the qPCR, 10 μl reactions were prepared in technical triplicate for each condition by mixing 1 μl of reverse-transcribed DNA, 4 μl of primer mix (to achieve a final concentration of 10 μM), and 5 μl of 2X SYBR Green PCR Master Mix (Applied biosystems cat# 4385612). For each gene target, the primer mixes consisted of the following forward (F) and reverse (R) primers: h18S (F: CGGCGACGACCCATTCGAAC; R: GAATCGAACCCTGATTCCCCGTC); hACTA2 (F: AAAAGACAGCTACGTGGGTGA; R: GCCATGTTCTATCGGGTACTTC); hCOL1A (F: ACGAAGACATCCCACCAATCACCT; R: AGATCACGTCATCGCACAACACCT); hFN1 (F: GGCAACGTGTTACGATGAT; R: CGGGAATCTTCTCTGTCAG). The qPCR was run on a ViiA 7 Real-Time PCR machine (Applied biosystems) using the following conditions:

Initial denaturation was done at 95°C for 20 s, then 40 cycles were repeated going from 1 s at 95°C to 20 s at 60°C.

Relative mRNA expression was calculated using the $2^{(-\Delta\Delta C_t)}$ method as described¹³³ using h18S as the housekeeping gene. The fold increase mRNA upon TGF β treatment was calculated by dividing each value from the previous step by the average mRNA expression of the control condition (without TGF β) for each target gene.

ERK1/2 phosphorylation assay—HEK293T cells were seeded and transfected with or without the G α_q ONE-GO biosensor as described in “BRET measurements in HEK293T cells”. One day after the transfection, cells were stimulated for 5 minutes with carbachol (100 μ M) with or without a 2 minute pre-incubation with YM-254890 (1 μ M) as indicated in Fig. S4B. Cells were harvested and centrifuged for 1 min at 14,000 $\times$ g and pellets stored at -20°C for subsequent immunoblot analysis (see “Protein electrophoresis and Immunoblotting” section below).

Protein electrophoresis and immunoblotting—Pellets of HEK293T, SH-SY5Y cells, or HeLa cells were resuspended on ice with lysis buffer (20 mM HEPES, 5 mM Mg(CH₃COO)₂, 125 mM K(CH₃COO), 0.4% (v:v) Triton X-100, 1 mM DTT, 10 mM β -glycerophosphate, 0.5 mM Na₃VO₄, supplemented with a protease inhibitor cocktail [SigmaFAST cat# S8830], pH 7.4) and thoroughly vortexed. Cardiac fibroblasts were lysed directly in the culture wells as described in “BRET measurements in primary cell cultures” with the same buffer and transferred to 1.5 ml tubes before thorough vortexing. Lysates were cleared by centrifugation (10 min at 14,000 $\times$ g, 4°C) and quantified by Bradford (Bio-Rad, cat#5000205). All samples were then boiled for 5 min in Laemmli sample buffer, except those used to detect PAR1 (Fig. 7C), which were incubated at 37°C for 10 min.

Proteins were separated by SDS-PAGE, followed by transfer to PVDF membranes (EMD Millipore cat# IPFL00010) for 2 h. Membranes were stained with Ponceau S solution (0.1% w/v Ponceau S in 5% v/v acetic acid) and imaged on a flatbed scanner prior to immunoblotting. PVDF membranes were blocked with TBS supplemented with 5% (w:v) non-fat dry milk for 1 hour, and then incubated sequentially with primary and secondary antibodies diluted in TBST supplemented with 2.5% (w:v) non-fat dry milk. Primary antibody species, vendors, and dilutions were as follows (in parenthesis): His (mouse, Sigma cat# H1029; 1:2,500); β -actin (rabbit, LI-COR cat# 926-42210; 1:2,500); GFP (mouse, Clontech cat# 632380; 1:2,000); FLAG (mouse, Millipore Sigma cat# F1804; 1:1000); Gas (rabbit, SCBT cat# sc-383; 1:500); tubulin (mouse, Sigma cat# T6074; 1:2,500); G β (mouse, SCBT cat# sc-166123; 1:250–1:500); PAR1 (mouse, Millipore Sigma cat# MABF244; 1:500); RFP (mouse, Invitrogen cat# MA5-15257; 1:2000); pERK1/2(T202/Y204) (rabbit, Cell Signaling cat# 4370; 1:1000); ERK1/2 (rabbit, Cell Signaling cat# 4695; 1:1000); G α_i3 (rabbit, Aviva cat# OAAB19207; 1:250), except in Fig. 3C, D, which has rabbit, Millipore Sigma cat# 06-270; 1:500; Nluc in Fig. 1D, Fig. S1A–B, Fig. S3B, Fig. S6 (rabbit, Promega⁴²; 1:1,000); Nluc in Fig. 2A, 3B, 4A, 7C (mouse, Promega cat# N700A; 1:500–1:1000); Nluc in Fig. 3C–D (mouse, R&D systems cat# MAB10026; 1:250). The following secondary antibodies were used at a 1:10,000 dilution (species and vendor indicated in parenthesis): anti-rabbit Alexa Fluor 680 (goat, Invitrogen cat# A21077); anti-mouse

Alexa Fluor 680 (goat, Invitrogen cat# A21058); anti-mouse IRDye 800 (goat, LI-COR cat# 926–32210); anti-rabbit DyLight 800 (goat, Thermo cat# 35571). Infrared imaging of immunoblots was performed according to manufacturer's recommendations using an Odyssey CLx infrared imaging system (LI-COR Biosciences). Images were processed using Image Studio software (LI-COR), and assembled for presentation using Photoshop and Illustrator software (Adobe).

QUANTIFICATION AND STATISTICAL ANALYSIS

Unless otherwise indicated, all experiments were performed at least three times, and presented as averages $\pm$ the standard error of the mean (SEM). For the sake of clarity, in the presentation of kinetic BRET measurements the SEM is only presented in the positive direction and displayed as bars of a lighter color tone than that of its corresponding data points. Protein structure images displayed in Fig. 2A, 2B were prepared in ICM (Molsoft LLC., San Diego, CA). Chemical structures (Fig. 5A, C) were generated using ChemDraw (PerkinElmer informatics, Waltham, MA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- ONE-GO is a platform of biosensors for all main classes of GPCR-activated G proteins
- ONE-GO sensors are broadly applicable across GPCRs, cell types, and assay formats
- ONE-GO sensors detect endogenous GPCR activity in many different primary cell types
- Context-dependence is a prevalent feature of endogenous GPCR activity

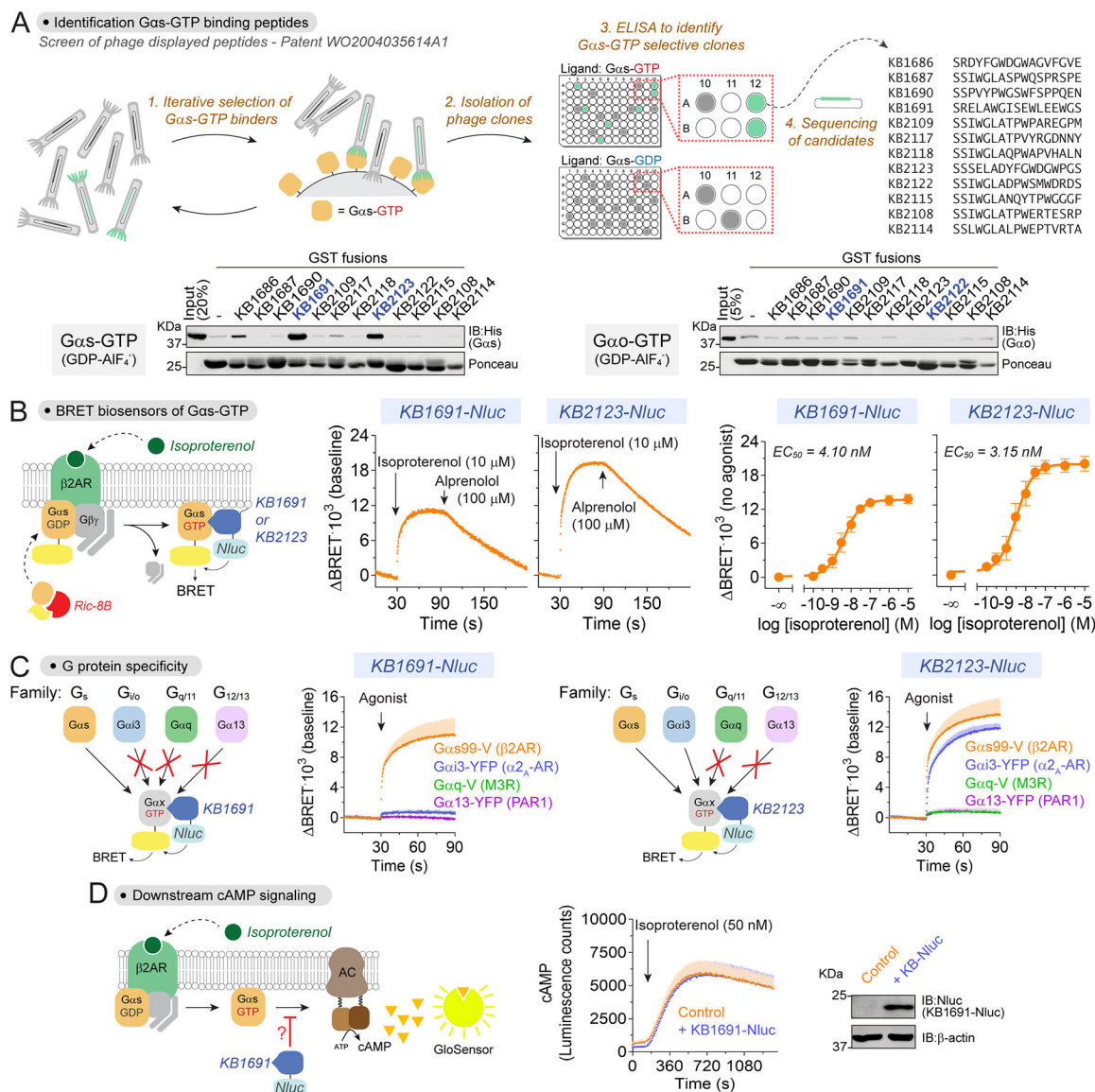


Figure 1. Direct detection of active Gas in cells with a BRET biosensor.

(A) Identification of Gas-GTP peptide binders. *Top*, schematic of phage display screen.

Bottom, pull-down of active, GDP-AlF₄⁻-loaded Gas or Gao with GST-fused peptides.

From n ≥ 2.

(B) Gas-GTP biosensor using KB1691 and KB2123 peptides. *Left*, BRET assay principle.

Center and left, BRET was measured in HEK293T cells expressing β2AR, Gas-99V, Gβγ and either KB1691-Nluc or KB2123-Nluc. A representative kinetic trace is shown on the center panels, and the mean ± S.E.M. (n=4) for the concentration-dependent curves on the right.

(C) Sensors based on KB1691, but not on KB2123, specifically detect Gas. BRET was measured in HEK293T cells expressing the indicated G protein / cognate GPCR pairs and either KB1691-Nluc or KB2123-Nluc. Cells were stimulated with either 10 μM

isoproterenol (G α s), 5 μ M brimonidine (G α i3), 100 μ M carbachol (G α q), or 30 μ M TRAP-6 (G α 13). Mean $\pm$ S.E.M. (n=3–4).

(D) KB1691 does not interfere with G α s-mediated cAMP production. Luminescence was measured in HEK293T cells expressing the cAMP probe Glosensor and exactly the same components as in panel B, except that KB1691-Nluc was omitted in the control. Mean $\pm$ S.E.M. (n=4).

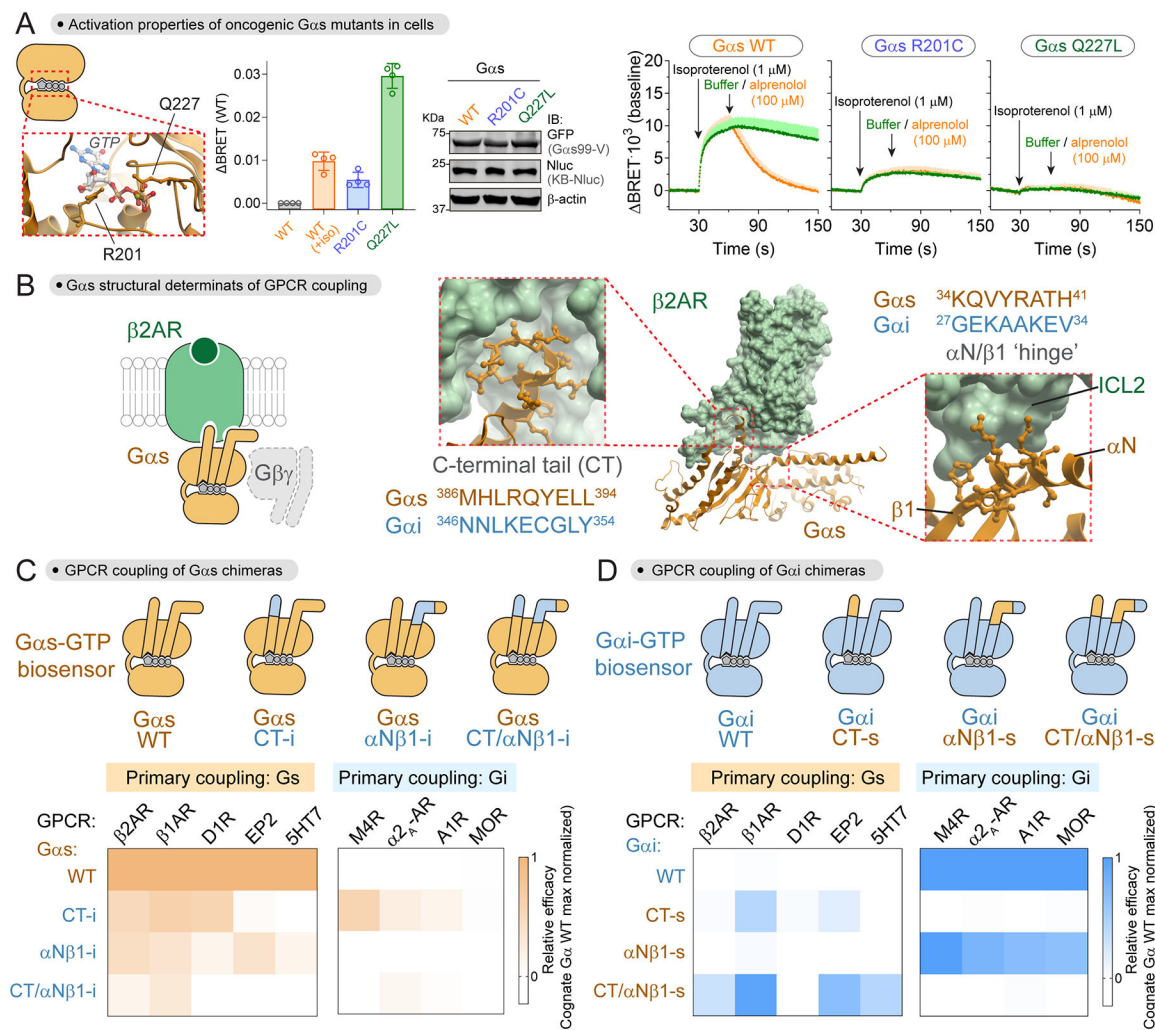


Figure 2. Mechanism of G α s activation by oncogenic mutations and of G α s coupling to GPCRs. (A) Activation properties of oncogenic G α s mutants. *Left*, view of G α s nucleotide-binding pocket (PDB: 1AZT). *Right*, BRET was measured in HEK293T cells expressing the same components as in Fig.1B with KB1691-Nluc and the indicated G α s-99V constructs. Bar graph represents BRET signal relative to unstimulated G α s-99V WT. Mean $\pm$ S.E.M. (n=4). Kinetic traces of cells expressing the same components are the difference in BRET from their corresponding unstimulated baselines. Mean $\pm$ S.E.M. (n=3). (B) View of structural elements of G α s involved in coupling to β 2AR based on their complex structure (PDB: 3SN6). (C, D) Contribution of the C-terminal tail (CT) and the α N/ β 1 'hinge' of G α subunits to their coupling to GPCRs. BRET was measured in HEK293T cells expressing the indicated YFP-tagged G α chimeras, GPCRs, and G $\beta\gamma$ with either a G α s-GTP biosensor (KB1691-Nluc, C) or a G α i-GTP biosensor (KB1753-Nluc, D). Heat maps correspond to the mean (n=3–4) of efficacy of the BRET responses detected relative to the maximal response observed with the cognate WT G protein. Full concentration-dependence curves in Fig.S2.

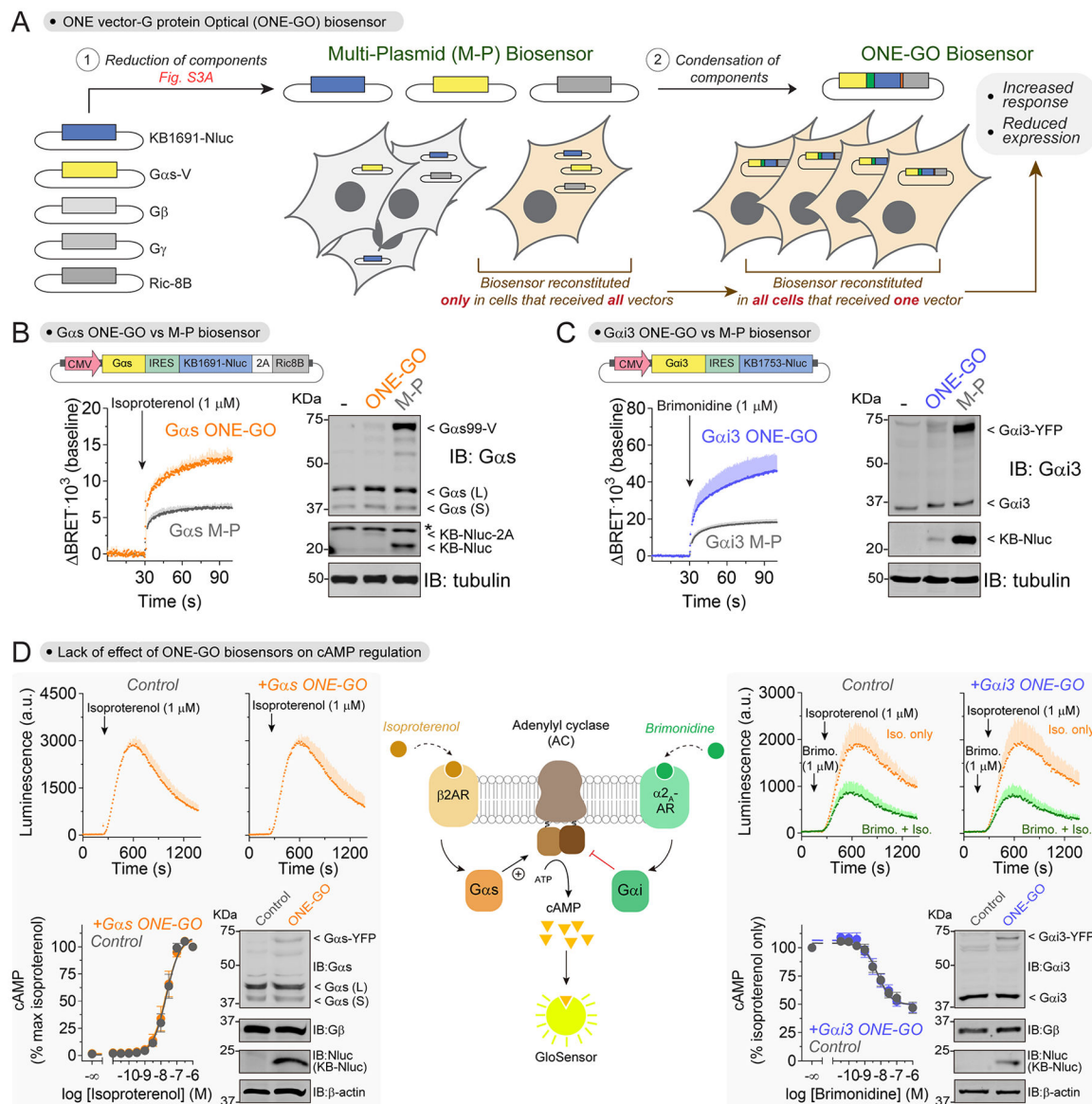


Figure 3. ONE vector G protein Optical (ONE-GO) biosensor designs display improved features.

(A) Schematic of the process to develop ONE-GO biosensors.

(B-C) Gαs and Gαi3 ONE-GO biosensor designs provide increased responses and reduced component expression. BRET was measured in HEK293T cells transfected with the indicated single-plasmid ONE-GO biosensors or their multi-plasmid (M-P) counterparts. β2AR or α_{2A}-AR were co-expressed for Gαs (**B**) or Gαi3 (**C**), respectively. Mean ± S.E.M. (n=3).

(D) ONE-GO biosensors do not interfere with GPCR-G protein signaling. Luminescence was measured in HEK293T cells expressing the cAMP probe GloSensor with or without the Gαs ONE-GO sensor (*Left*, endogenous β2AR) or Gαi3 ONE-GO sensor (*Right*, exogenous α_{2A}-AR). Mean ± S.E.M. (n=3). Immunoblots show that ONE-GO sensors do not change the levels of endogenous G protein subunits.

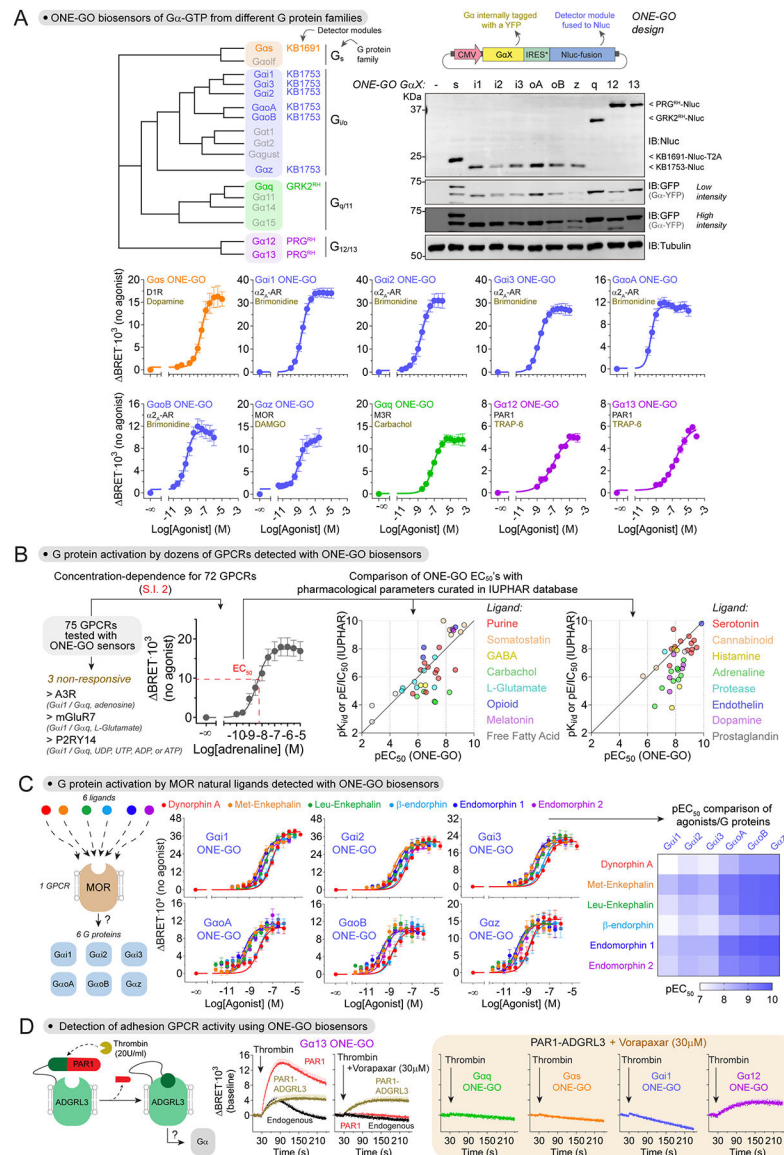


Figure 4. ONE-GO biosensors report activation across G protein families and for many GPCRs. (A) Ten ONE-GO biosensors report activity across all G protein families. *Top*, dendrogram of Gα subunits with their corresponding detector modules used in ONE-GO designs, and expression of biosensor components in HEK293T cells ($n \geq 3$). *Bottom*, BRET in HEK293T cells expressing the indicated ONE-GO biosensors and GPCRs. Mean $\pm$ S.E.M. ($n=3-5$). (B) ONE-GO biosensors report the activity of dozens of GPCRs. Concentration-dependent BRET responses were measured in HEK293T cells for 75 GPCRs to determine EC_{50} values, which were plotted against curated pharmacological parameters (pK_d , pK_i , or pE/IC_{50}) available in the IUPHAR database. (C) Activation profile of G proteins of the $G_{i/o}$ family with ONE-GO biosensors upon stimulation of the MOR with six natural neuropeptide ligands. BRET in HEK293T cells expressing the MOR and the indicated ONE-GO biosensors upon stimulation

with dynorphin A, Met-Enkephalin, Leu-Enkephalin, β -endorphin, endomorphin 1, or endomorphin 2. Mean $\pm$ S.E.M. (n=5–6). The heatmap shows mean pEC₅₀ values.

(D) Profiling the activation of different G proteins by the adhesion GPCR ADGRL3 using ONE-GO biosensors. *Left*, PAR1-ADGRL3 chimera activated by thrombin. *Middle and Right*, BRET in HEK293T cells expressing the indicated ONE-GO sensors and either the PAR1 receptor or the PAR1-ADGRL3 chimera upon stimulation with thrombin. The PAR1 antagonist vorapaxar was used to blunt G protein activation by endogenously expressed PAR1 receptors. Mean $\pm$ S.E.M. (n=3–4).

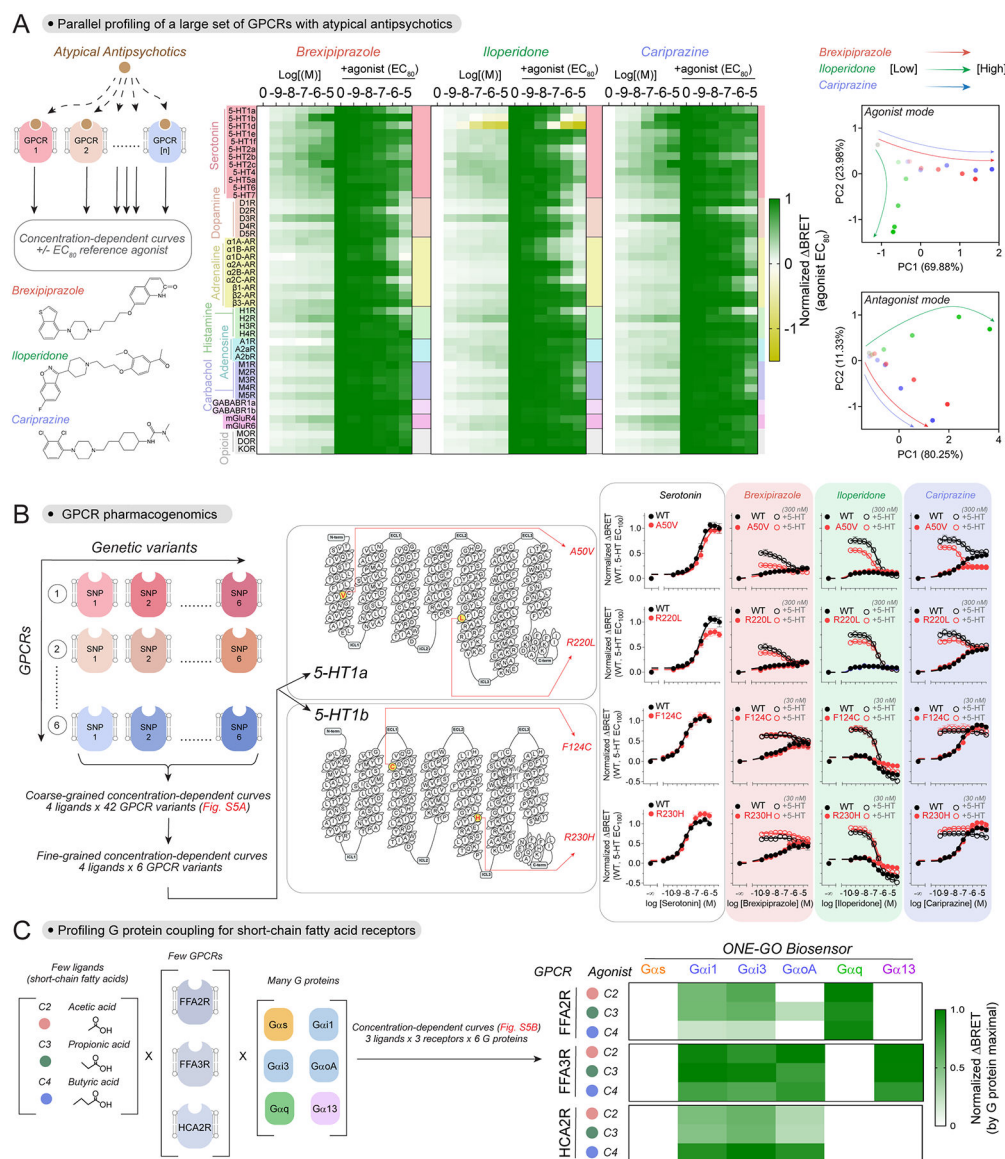


Figure 5. Large-scale parallel interrogation of GPCR activity with ONE-GO biosensors. (A) Parallel profiling of atypical antipsychotics across a large set of receptors. *Left*, schematic of the assay and structure of the compounds investigated. *Middle*, BRET in HEK293T cells expressing the indicated GPCRs along with a cognate ONE-GO biosensor upon stimulation with the indicated concentrations of each antipsychotic alone or in presence of an agonist at its EC₈₀ concentration (see [S15] for details). Mean (n=3–7). *Right*, principal component analysis (PCA) of the data presented in the heatmaps. (B) Pharmacogenomic profiles of atypical antipsychotics. *Left*, schematic of the assay (coarse-grained curves shown in Fig.S5A). *Middle*, Snake plots for 5-HT_{1a} and 5-HT_{1b} showing genetic variants investigated on the right. *Right*, BRET in HEK293T cells expressing the indicated GPCRs and the Gαi1 ONE-GO sensor. The indicated compounds were tested by themselves (filled circles) or in presence of 5-HT (open circles). Mean ± S.E.M. (n=3–4).

(C) Profiling of G protein selectivity across short-chain fatty acid receptors. *Left*, schematic of variables investigated. *Right*, BRET in HEK293T cells expressing the indicated GPCR / ONE-GO biosensor combinations upon stimulation with the indicated agonist. Results are maximal responses normalized by biosensor (mean, n=3–6). Full concentration-dependent curves in Fig.S5B.

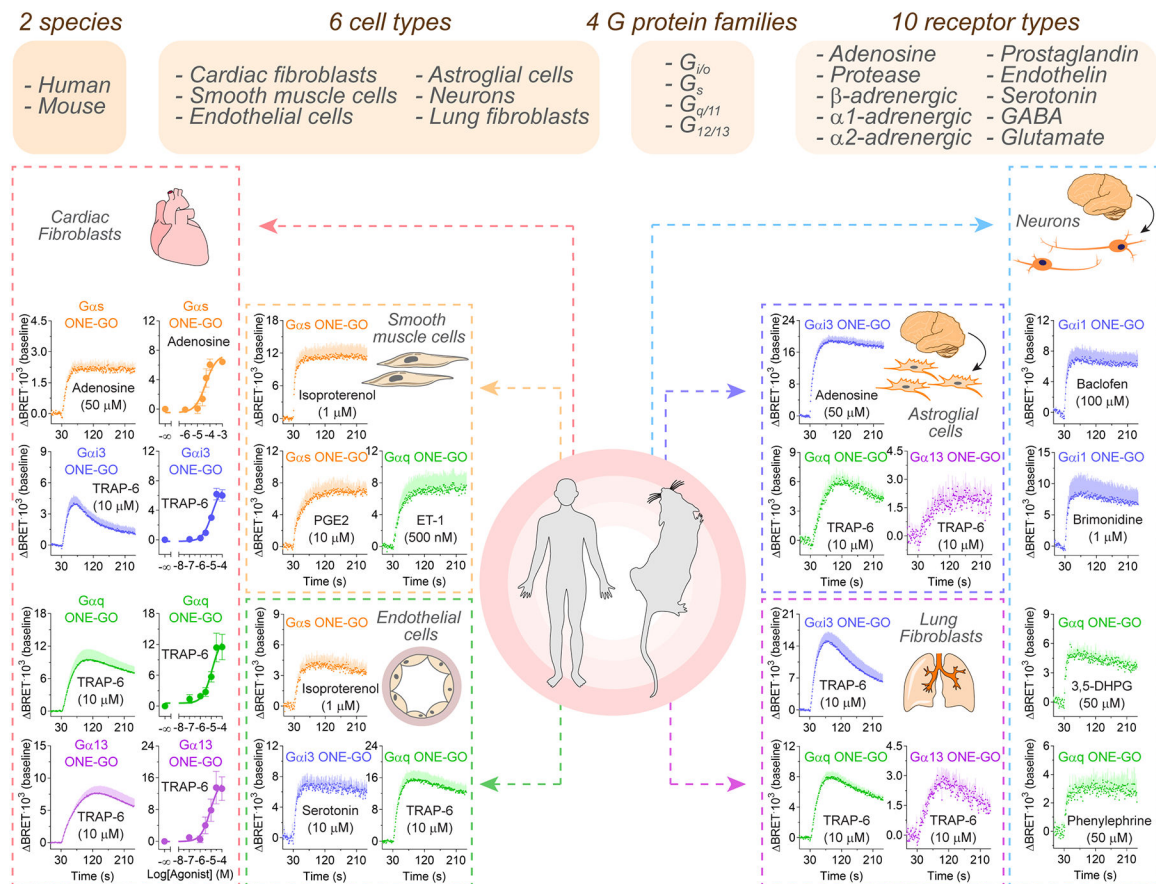


Figure 6. Detection of endogenous GPCR activity across a wide palette of primary cells with ONE-GO biosensors.

Top, summary of responses triggered by endogenous GPCRs detected with ONE-GO biosensors for all G protein families in multiple human and mouse primary cells of different origins. *Bottom*, BRET responses were measured in the indicated primary cells transduced with ONE-GO biosensors. Mean $\pm$ S.E.M. (n=3–6). Additional examples and controls in Fig.S7.

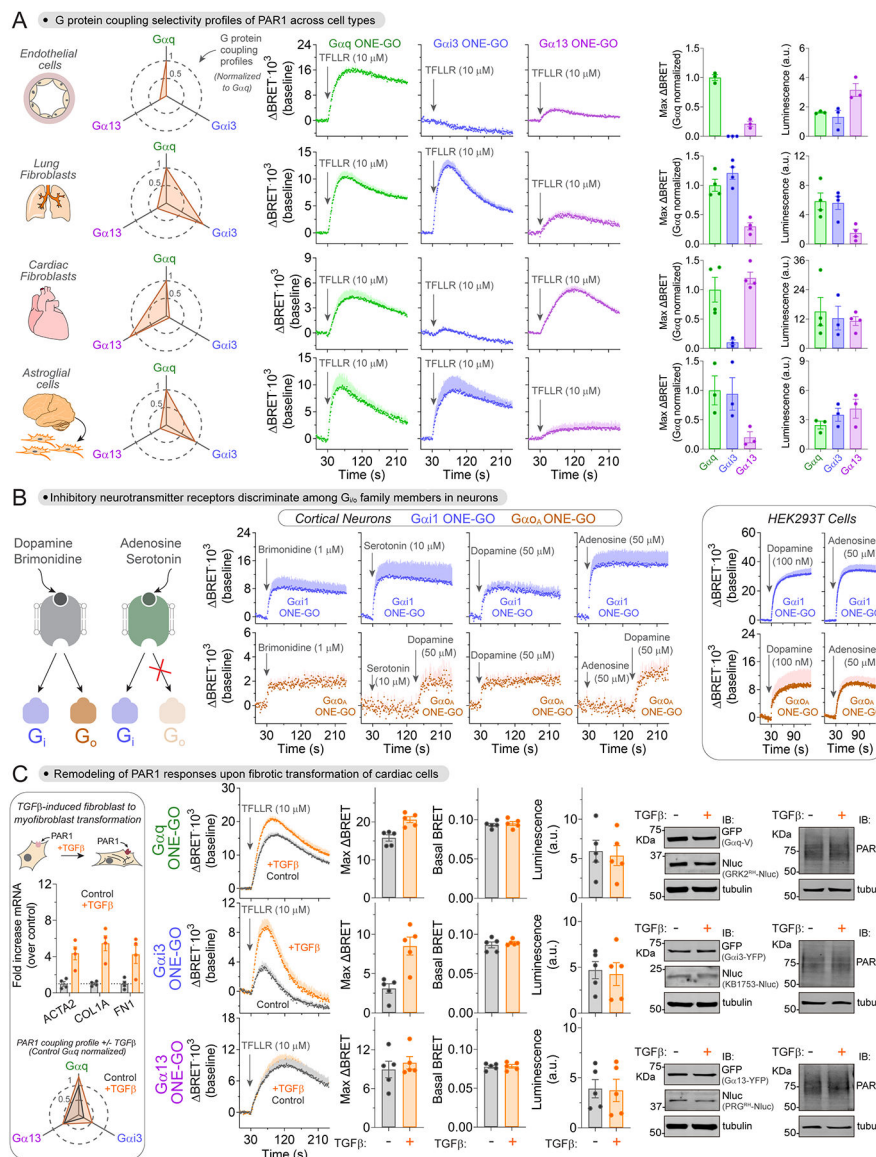


Figure 7. ONE-GO biosensors reveal context-dependent activity of endogenous GPCRs.
(A) Cell type-dependent G protein selectivity profiles of PAR 1. *Left*, spider plot summarizing PAR1 G protein activation profiles for each cell type. *Middle*, BRET in each one of the 4 primary cell types transduced with the indicated ONE-GO biosensor. *Right*, maximal BRET responses normalized to Gαq ONE-GO and raw luminescence counts indicative of biosensor expression. Mean ± S.E.M. (n=3–4).
(B) Discrimination across G_{i/o} isoforms by neuroinhibitory GPCRs in primary neurons. *Left*, BRET in primary mouse cortical neurons transduced with Gαi1 ONE-GO (top row, blue) or GαoA ONE-GO (bottom row, brown) biosensors stimulated with the indicated neurotransmitters. Mean ± S.E.M. (n=3–6). *Right (box)*, BRET in HEK293T cells expressing either the D2R or the A1R along with the indicated ONE-GO biosensor. Mean ± S.E.M. (n=3).

(C) Myofibroblast transformation remodels the G protein selectivity profile of PAR1. *Left (box)*, confirmation of TGF β -induced myofibroblast transformation by RT-qPCR (mean $\pm$ S.E.M., n=4), and spider plot summarizing PAR1 G protein activation profiles before and after myofibroblast transformation (mean, n=5). *Middle*, BRET in human cardiac fibroblasts transduced with the indicated ONE-GO biosensors and treated (orange) or not (grey) with TGF β . Bar graphs represent from left to right: maximal BRET responses normalized to G α_q ONE-GO, BRET signal in unstimulated cells, and raw luminescence counts indicative of biosensor expression. Mean $\pm$ S.E.M. (n=5). *Right*, immunoblots showing no difference in expression of the biosensor components (G α , Nluc) or PAR1 upon TGF β treatment (from n=3).

Key Resources Table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti His	Millipore Sigma	Cat #H1029
Rabbit monoclonal anti β -Actin	LI-COR	Cat #926–42210
Mouse monoclonal anti GFP (JL-8)	Clontech	Cat #632380
Mouse monoclonal anti FLAG (M2)	Millipore Sigma	Cat #F1804
Rabbit polyclonal anti Gas (C18)	SCBT	Cat #sc-383
Mouse monoclonal anti α -tubulin	Millipore Sigma	Cat #T6074
Mouse monoclonal anti G β (H-1)	SCBT	Cat #sc-166123
Mouse monoclonal anti PAR-1 (ATAP2)	Millipore Sigma	Cat #MABF244
Rabbit polyclonal anti Gai3	Aviva Systems Biology	Cat #OAB19207
Rabbit polyclonal anti Gai3	Millipore Sigma	Cat #06–270
Rabbit polyclonal anti Nanoluciferase	Promega ⁴²	N/A
Mouse monoclonal anti Nanoluciferase	Promega	Cat #N7000A
Mouse monoclonal anti Nanoluciferase	R&D Systems	Cat #MAB10026
Mouse monoclonal anti RFP	Invitrogen	Cat #MA5–15257
Rabbit monoclonal anti pERK1/2 (T202/Y204)	Cell Signaling	Cat #4370
Rabbit monoclonal anti ERK1/2	Cell Signaling	Cat #4695
Goat anti rabbit Alexa Fluor 680	Invitrogen	Cat #A-21077
Goat anti mouse Alexa Fluor 680	Invitrogen	Cat #A21058
Goat anti mouse IRDye 800	LI-COR	Cat #926–32210
Goat anti rabbit DyLight 800	Thermo	Cat #35571
Bacterial and virus strains		
NEB 5-alpha competent <i>E. coli</i>	NEB	Cat #C2987I
Chemicals, peptides, and recombinant proteins		
Isoproterenol	Alfa Aesar	Cat #J61788
Alprenolol	Tocris Bioscience	Cat #2806
Brimonidine	Ark Pharm	Cat #AK-35795
Yohimbine	Alfa Aesar	Cat #J60185
Carbachol	Acros Organics	Cat #108240050
TRAP-6	Anaspec	Cat #AS-24190
Thrombin	Prolytix	Cat #HCT-0020
Vorapaxar	MCE	Cat #HY-10119
Dopamine	Alfa Aesar	Cat #A11136
Haloperidol	Alfa Aesar	Cat #J61688
PGE2	TCI	Cat #P1884
Serotonin	Alfa Aesar	Cat #B21263
Adenosine	Acros Organics	Cat #164045000

REAGENT or RESOURCE	SOURCE	IDENTIFIER
CGS 15943	Sigma	Cat #C199
GS 6201	Cayman Chemical	Cat #33333
KW 3902	Cayman Chemical	Cat #11940
DAMGO	BACHEM	Cat #4007829
Dynorphin A	Cayman Chemical	Cat #18169
Met-Enkephalin	Cayman Chemical	Cat #23284
Leu-Enkephalin	Cayman Chemical	Cat #23283
β -endorphin	Cayman Chemical	Cat #24955
Endomorphin 1	Cayman Chemical	Cat #23280
Endomorphin 2	Cayman Chemical	Cat #23281
Naloxone	Alfa Aesar	Cat #J60013
Adrenaline	Alfa Aesar	Cat #L04911
Histamine	Alfa Aesar	Cat #J61727
Anandamide	ENZO life sciences	Cat #BML-FA017
Endothelin-1	Millipore Sigma	Cat #05-23-3800
UDP	MP Biomedicals	Cat #ICN101205
UTP	Fisher	Cat #J63427.MC
ADP	Fisher	Cat #AAJ6449706
ATP	Fisher	Cat #AAL1452206
L-Glutamate	Acros Organics	Cat #156210010
Somatostatin-14	Fisher	Cat #AAJ66168LB0
DynorphinA	Cayman Chemical	Cat #18169
Melatonin	Fisher	Cat #AC125360050
GABA	Tocris Bioscience	Cat #0344
CGP-54626	Tocris Bioscience	Cat #1088
Acetic acid	Acros Organics	Cat #212662500
Propionic acid	Acros Organics	Cat #149010250
Butyric acid	Acros Organics	Cat #263190250
Brexipiprazole	Cayman Chemical	Cat #22906
Iloperidone	Cayman Chemical	Cat #22957
Cariprazine	Cayman Chemical	Cat #31446
Baclofen	Tocris Bioscience	Cat #0796
3,5-DHPG	Tocris Bioscience	Cat #0805
Phenylephrine	Tocris Bioscience	Cat #2838
TFLLR	Cayman Chemical	Cat #27548
Pertussis toxin	List Biological Labs	Cat #179 A
YM-254890	FUJIFILM Wako Pure Chemical Corp.	Cat #257-00631
TGF β 1	R&D Systems	Cat #7666-MB
Poly-L-Lysine hydrobromide	Millipore Sigma	Cat #P9155

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Polybrene	Tocris Bioscience	Cat #7711/10
Critical commercial assays		
Nano-Glo Luciferase Assay System	Promega	Cat #N1120
LunaScript RT SuperMix Kit	NEB	Cat #E3010
Fast SYBR Green Master Mix	Applied Biosystems	Cat #4385612
QuikChange II Site-Directed Mutagenesis Kit	Agilent	Cat # 200523
Deposited data		
ONE-GO Biosensors Kit and related plasmids (including full plasmid sequences)	Addgene	Kit #1000000224
Original images from blotting	This paper	Mendeley: http://dx.doi.org/10.17632/9tjcy8rg4t.1
Experimental models: Cell lines		
HEK293T	ATCC	Cat #CRL-3216
HeLa	ATCC	Cat #CCL-2
SH-SY5Y	ATCC	Cat #CRL-2266
Lenti-X 293T	Takara Bio	Cat #632180
Experimental models: Organisms/strains		
Mouse: C57BL/6N	MMRRC	N/A
Oligonucleotides		
F-h18S (CGGCGACGACCCATTCTGAAC)	This paper	N/A
R-h18S (GAATCGAACCCTGATCCCCGTC)	This paper	N/A
F-hACTA2 (AAAAGACAGCTACGTGGGTGA)	This paper	N/A
R-hACTA2 (GCCATGTTCTATCGGGTACTTC)	This paper	N/A
F-hCOL1A (ACGAAGACATCCCACCAATCACCT)	This paper	N/A
R-hCOL1A (AGATCACGTCATCGCACAAACACCT)	This paper	N/A
F-hFN1 (GGCAACGTGTTACGATGAT)	This paper	N/A
R-hFN1 (CGGGAATCTTCTCTGTCAG)	This paper	N/A
Recombinant DNA		
pcDNA3.1-mas-KB1691-Nluc	This paper	N/A
pcDNA3.1-mas-KB2123-Nluc	This paper	N/A
pcDNA3.1-mas-GRK3ct-NLuc	27	N/A
pcDNA3.1-mas-KB1753-Nluc	30	N/A
pcDNA3.1-mas-GRK2 ^{RH} -Nluc	30	N/A
pcDNA3.1-mas-PRG ^{RH} -Nluc	30	N/A
pcDNA3.1-Gαs-67V	38	N/A
pcDNA3.1-Gαs-99V	38	N/A
pcDNA3.1-Gαs-154V	38	N/A
pcDNA3.1-mas-KB1691-mKate2	This paper	N/A
pcDNA3.1-mas-KB1753-mKate2	This paper	N/A
pcDNA3.1-Gαs-99V R201C	This paper	N/A

REAGENT or RESOURCE	SOURCE	IDENTIFIER
pcDNA3.1-Gas-99V Q227L	This paper	N/A
pcDNA3.1-Gai3-YFP	121	N/A
pcDNA3.1-Gaq-V	101	N/A
pcDNA3.1-Ga13-YFP	30	N/A
pcDNA3.1-Gas-99V CT-i	This paper	N/A
pcDNA3.1-Gas-99V α N β 1-i	This paper	N/A
pcDNA3.1-Gas-99V CT-i/ α N β 1-i	This paper	N/A
pcDNA3.1-Gai3-YFP CT-s	This paper	N/A
pcDNA3.1-Gai3-YFP α N β 1-s	This paper	N/A
pcDNA3.1-Gai3-YFP CT-s/ α N β 1-s	This paper	N/A
pcDNA3.1-FLAG-Ric-8B	122	N/A
pcDNA3.1-G β i	26	N/A
pcDNA3.1-G γ 2	26	N/A
pcDNA3.1-G β 1-V(155–239)	26	N/A
pcDNA3.1-G γ 2-V(1–155)	26	N/A
Glosensor 22F	Promega	Cat #E2301
pcDNA3.1-cyto-KB1691-Nluc	This paper	N/A
pVenus-C1-Venus-K-Ras	41	N/A
pLVX-CMV-Gas-99V-IRES*-KB1691-Nluc-T2A-Ric-8B	This paper	N/A
pLVX-CMV-Gai1-YFP-IRES*-KB1753-Nluc	This paper	N/A
pLVX-CMV-Gai2-YFP-IRES*-KB1753-Nluc	This paper	N/A
pLVX-CMV-Gai3-YFP-IRES*-KB1753-Nluc	This paper	N/A
pLVX-CMV-GaoA-YFP-IRES*-KB1753-Nluc	This paper	N/A
pLVX-CMV-GaoB-YFP-IRES*-KB1753-Nluc	This paper	N/A
pLVX-CMV-Gaz-YFP-IRES*-KB1753-Nluc	This paper	N/A
pLVX-CMV-Gaq-V-IRES*-GRK2RH-Nluc	This paper	N/A
pLVX-CMV-Ga12-YFP-IRES*-PRGRH-Nluc	This paper	N/A
pLVX-CMV-Ga13-V-IRES*-PRGRH-Nluc	This paper	N/A
pLVX-SV40-Gas-99V-IRES*-KB1691-Nluc-T2A-Ric-8B	This paper	N/A
pLVX-SV40-Gai3-YFP-IRES*-KB1753-Nluc	This paper	N/A
pLVX-CMV-IR ES ^{WT} -GRK3ct-Nluc-T2A-G β 1-T2A-G γ 2-V	This paper	N/A
pLVX-CMV-Gas-IRES ^{WT} -GRK3ct-Nluc-T2A-G β 1-T2A-G γ 2-V	This paper	N/A
pLVX-CMV-Gai3-IRES ^{WT} -GRK3ct-Nluc-T2A-G β 1-T2A-G γ 2-V	This paper	N/A
pLVX-hSyn-Gai1-YFP-IRES*-KB1753-Nluc	This paper	N/A
pLVX-hSyn-GaoA-YFP-IRES*-KB1753-Nluc	This paper	N/A
pLVX-hSyn-Gaq-V-IRES*-GRK2RH-Nluc	This paper	N/A
pLVX-CMV-Gai3 G183S-YFP-IRES*-KB1753-Nluc	This paper	N/A
pSPAX2	Addgene	#12260

REAGENT or RESOURCE	SOURCE	IDENTIFIER
pMD2.G	Addgene	#12259
pcDNA3.1-FLAG-HTR1A (human)	123	N/A
pcDNA3.1(+)-HTR1B (human)	This paper	N/A
pcDNA3.1(+)-HTR1D (human)	This paper	N/A
pcDNA3.1(+)-HTR1E (human)	This paper	N/A
pcDNA3.1(+)-HTR1F (human)	This paper	N/A
pcDNA3.1-FLAG-HTR2A (human)	123	N/A
pcDNA3.1(+)-HTR2B (human)	This paper	N/A
pcDNA3.1(+)-HTR2C (human)	This paper	N/A
pcDNA3.1(+)-HTR4 (human)	This paper	N/A
pcDNA3.1(+)-HTR5 (human)	This paper	N/A
pcDNA3.1(+)-HTR6 (human)	This paper	N/A
pcDNA3.1(+)-HTR7A (human)	cDNA Resource Center	Cat #HTR07A0000
pcDNA3.1(+)-DRD1 (human)	cDNA Resource Center	Cat #DRD0100000
pcDNA3.1(+)-FLAG-D2DR (human)	124	N/A
pcDNA3.1-FLAG-D3R (human)	125	N/A
pcDNA3.1-FLAG-D4R (human)	126	N/A
pcDNA3.1-FLAG-D5R (human)	127	N/A
pcDNA3.1–3xHA-ADRA1A (human)	cDNA Resource Center	Cat #AR0A1ATN00
pcDNA3.1(+)-ADRA1B (human)	This paper	N/A
pcDNA3.1(+)-ADRA1D (human)	This paper	N/A
pcDNA3- α 2A/D-AR (rat)	128,129	N/A
pcDNA3.1(+)-ADRA2B (human)	This paper	N/A
pcDNA3.1(+)-ADRA2C (human)	This paper	N/A
pcDNA3-Flag-beta-1-adrenergic receptor (human)	Addgene ¹³⁰	Cat #14698
pcDNA3-Flag-beta-2-adrenergic receptor (human)	Addgene ¹³⁰	Cat #14697
pcDNA3.1(+)-ADRB3 (human)	This paper	N/A
pcDNA3.1(+)-H1R (human)	This paper	N/A
pcDNA3.1(+)-H2R (human)	This paper	N/A
pcDNA3.1(+)-H3R (human)	This paper	N/A
pcDNA3.1(+)-H4R (human)	This paper	N/A
pcDNA3.1(+)-CB1 (human)	This paper	N/A
pcDNA3.1(+)-CB2 (human)	This paper	N/A
pBJ-FLAG-hPAR1 (human)	Addgene ¹³¹	Cat #53226
pcMIM-ETAR (human)	132	N/A
pcDNA3.1(+)-PTGER2 (human)	cDNA Resource Center	Cat #PER0200000
pcDNA3.1(+)-ADORA1 (human)	cDNA Resource Center	Cat #ADRA100000
pcDNA3.1(+)-ADORA2A (human)	cDNA Resource Center	Cat #ADRA2ATN00
pcDNA3.1(+)-ADORA2B (human)	This paper	N/A

REAGENT or RESOURCE	SOURCE	IDENTIFIER
pcDNA3.1(+)-A3R (human)	This paper	N/A
pcDNA3.1(+)-P2Y1 (human)	This paper	N/A
pcDNA3.1(+)-P2Y2 (human)	This paper	N/A
pcDNA3.1(+)-P2Y4 (human)	This paper	N/A
pcDNA3.1(+)-P2Y6 (human)	This paper	N/A
pcDNA3.1(+)-P2Y11 (human)	This paper	N/A
pcDNA3.1(+)-P2Y12 (human)	This paper	N/A
pcDNA3.1(+)-P2Y13 (human)	This paper	N/A
pcDNA3.1(+)-P2Y14 (human)	This paper	N/A
pcDNA3.1(+)-GRM1 (human)	This paper	N/A
pcDNA3.1(+)-GRM2 (human)	This paper	N/A
pRK5-GRM3 (rat)	¹³³ (Gifted from J. Levitz)	N/A
pcDNA3.1(+)-GRM4 (human)	cDNA Resource Center (Gifted from J. Wang)	Cat #GRM4000000
pcDNA3.1(+)-GRM5 (human)	This paper	N/A
pcDNA3.1(+)-GRM6 (human)	cDNA Resource Center	Cat #GRM6000000
pcDNA3.1(+)-GRM7 (human)	This paper	N/A
pcDNA3.1(+)-GRM8 (human)	This paper	N/A
pcDNA3.1(+)-SSTR1 (human)	This paper	N/A
pcDNA3.1(+)-SSTR2 (human)	This paper	N/A
pcDNA3.1(+)-SSTR3 (human)	This paper	N/A
pcDNA3.1(+)-SSTR4 (human)	This paper	N/A
pcDNA3.1(+)-SSTR5 (human)	This paper	N/A
Myc-M1R (human)	¹²⁷	N/A
pcDNA3.1(+)-CHRM2 (human)	This paper	N/A
pcDNA3.1(+)-3xHA-M3R (human)	cDNA Resource Center	Cat #MAR030TN00
pcDNA3.1(+)-2xHA-hM4R (human)	cDNA Resource Center	Cat #MAR040TN00
pcDNA3.1(+)-CHRM5 (human)	This paper	N/A
pcDNA3.1-HA-mMOR (mouse)	¹³⁴	N/A
pcDNA3.1(+)-DOR (human)	cDNA Resource Center	Cat #OPRD100000
pcDNA3.1(+)-KOR (human)	cDNA Resource Center	Cat #OPRK100000
pcDNA3.1(+)-MT1 (human)	This paper	N/A
pcDNA3.1(+)-MT2 (human)	This paper	N/A
pcDNA3.1(+)-GABAb R1a (rat)	¹³⁵	N/A
pcDNA3.1(+)-GABAb R1b (rat)	¹³⁵	N/A
pcDNA3.1(+)-GABAb R2 (rat)	¹³⁵	N/A
pcDNA3.1(+)-HCA2 (human)	This paper	N/A
pcDNA3.1(+)-FFAR2 (human)	This paper	N/A
pcDNA3.1(+)-FFAR3 (human)	This paper	N/A

REAGENT or RESOURCE	SOURCE	IDENTIFIER
pcDNA3.1(+)-PAR1(Nterm)-ADGRL3 (mouse)	66	N/A
Software and algorithms		
GraphPad Prism	GraphPad Software	https://www.graphpad.com/scientific-software/prism/
Adobe Photoshop	Adobe	https://www.adobe.com/
Adobe Illustrator	Adobe	https://www.adobe.com/
ICM	Molsoft LLC	http://www.molsoft.com/
Other		
5 mm diameter glass coverslips	World Precision Instruments	Cat #502040