JBC Papers in Press. Published on December 28, 2018 as Manuscript RA118.006231
The latest version is at http://www.jbc.org/cgi/doi/10.1074/jbc.RA118.006231

A dynamic and screening-compatible nanoluciferase-based complementation assay enables profiling of individual GPCR-G protein interactions

Céline Laschet[1], Nadine Dupuis[1], and Julien Hanson[1,2*]

From the [1]Laboratory of Molecular Pharmacology, GIGA-Molecular Biology of Diseases, ULiège; [2] Laboratory of Medicinal Chemistry, CIRM-Drug target and Lead Discovery, ULiège, Belgium CHU, B34 (+4), 11 avenue de l'hôpital, B-4000 Liège, Belgium

Running Title: *Real-time GPCR-G protein interaction assay*

[*]To whom correspondence should be addressed: Julien Hanson: Laboratory of Molecular Pharmacology, GIGA, ULiège, CHU, B34 (+4), 11 avenue de l'hôpital, B-4000 Liège, Belgium; j.hanson@uliege.be; Tel. +32 (0) 4 3664748; Fax. +32 (0) 4 3664198.

Keywords: G protein-coupled receptor (GPCR), G protein, high-throughput screening (HTS), drug screening, pharmacology, functional selectivity, biased signaling, complementation assay, dopamine, Nanoluciferase

ABSTRACT

G protein-coupled receptors (GPCRs) are currently the target of more than 30% of the marketed medicines. However, there is an important medical need for ligands with improved pharmacological activities on validated drug targets. Moreover, most of these ligands remain poorly characterized, notably because of a lack of pharmacological tools. Thus, there is an important demand for innovative assays that can detect and drive the design of compounds with novel or improved pharmacological properties. In particular, a functional and screening-compatible GPCR-G protein interaction assay is still unavailable. Here, we report on a nanoluciferase-based complementation technique to detect ligands that promote a GPCR-G protein interaction. We demonstrate that our system can be used to profile compounds with regard to the G proteins they activate through a given GPCR. Furthermore, we established a proof of applicability of screening for distinct G proteins on dopamine receptor D_2 whose differential coupling to $G\alpha_{i/o}$ family members has been extensively studied. In a D_2-$G\alpha_{i1}$ versus D_2-$G\alpha_o$ screening, we retrieved five agonists that are currently being used in antiparkinsonian medications. We determined that in this assay, piribedil and pergolide are full agonists for the recruitment of $G\alpha_{i1}$ but are partial agonists for $G\alpha_o$, that the agonist activity of ropinirole is biased in favor of $G\alpha_{i1}$ recruitment, and that the agonist activity of apomorphine is biased for $G\alpha_o$. We proposed that this newly developed assay could be used to develop molecules that selectively modulate a particular G protein pathway.

G protein-coupled receptors (GPCRs) are a large family of membrane proteins that have pivotal functions in physiology and are directly targeted by more than 30% of our therapeutic arsenal (1). Given their successes in drug discovery, it is common sense to postulate that uncharacterized members hold great potential in terms of innovative therapeutic strategies (2-5). Several authors recently proposed that the paucity of adequate pharmacological tools was precluding research on elusive receptors (5, 6). In addition, there is still an unmet medical need in various diseases for drugs with improved properties such as increased potency, higher selectivity or refined efficacy for existing validated drug targets. Thus, the drug discovery process would benefit from more sophisticated assays able to detect with maximal accuracy the ligands with a desired pharmacological profile.

GPCR signaling has been extensively studied and is notoriously complex. The current paradigm states that the binding of a ligand to its receptor stabilizes active conformations that in turn triggers through allosteric effects the formation of an active

complex bound with intracellular partners (7). It is generally accepted that the prime event following ligand binding is the interaction of the active receptor with heterotrimeric G proteins composed of α and βγ subunits (8). These elements dissociate upon activation and each of them has the capacity to promote distinct signaling pathways (9). Following G protein activation, the receptor undergoes desensitization and internalization through diverse processes involving phosphorylation by GPCR-specific kinases (GRK) and scaffolding by proteins such as arrestins (10).

In humans, the G protein family comprises 16 members that are classified according to the identity of their α subunits into 4 families (11). $G\alpha_s$ family ($G\alpha_{slong}$, $G\alpha_{sshort}$, $G\alpha_{olf}$) increases while $G\alpha_{i/o}$ family ($G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_o$, $G\alpha_z$) decreases adenylate cyclase (AC) activity. Hence, $G\alpha_{s/olf}$ and $G\alpha_{i/o}$ oppositely regulate levels of cAMP in the cell (9). $G\alpha_{q/11}$ family ($G\alpha_q$, $G\alpha_{11}$, $G\alpha_{15}$, $G\alpha_{16}$) triggers notably the activation of the Phospholipase C-β and calcium mobilization through the release of DAG and IP_3 (9). $G\alpha_{12/13}$ ($G\alpha_{12}$ & $G\alpha_{13}$) activate the small GTPases regulators Rho-GEF (9). Although individual receptors were initially seen as being selective for a given pathway and thus coupled to a single G protein, it has been observed that most receptors display at least some level of promiscuousness toward different G proteins or G protein families (9). Another layer of complexity exists in GPCR signaling with the observation of functional selectivity, which can be defined as the ability of a ligand to stabilize a specific receptor conformation leading to a unique and ligand-determined profile of signaling pathways activation (7).

A large array of pharmacological assays has been developed for the identification of substances able to modulate G proteins through GPCRs. Most of them focus on the downstream events triggered by G protein activation such as the generation of cAMP ($G\alpha_{s/olf}$ & $G\alpha_{i/o}$) (12, 13), Ca^{2+} (14) and IP_1 ($G\alpha_{q/11}$), downstream activation of gene promoters or more recently shedding of TGF-α ($G\alpha_{q/11}$ & $G\alpha_{12/13}$) (15). More generic and holistic assays measuring the accumulation of the non-hydrolysable GTP-γ-S (reflecting G protein activation) (16) or cell morphology (17) have also been extensively used. In order to extent the scope of an assay to more than one or two pathways, promiscuous G proteins linking most receptors to common second messengers are generally added to the system (18). Collectively, these assays are limited in the way that they cannot give direct information on the kind of G protein, or even on the identity of the GPCR, that has been activated by a given ligand. Thus, during screening campaigns, they are prone to deliver a high rate of false positives (13).

More recent techniques based on resonance energy transfer (BRET and FRET) have given unprecedented access to the study of individual interaction between G protein and their receptors in living cells (19). However, these approaches suffer from important drawbacks such as limited sensitivity (BRET) or high background noise (FRET) that limit their use, for instance in high throughput screenings (20). In addition, they require the presence of bulky donor and/or acceptors, although reduced-size donors such as NanoLuc (BRET (21)) or FlasH (FRET (22)) are now routinely used.

Here we describe a simple and flexible nanoluciferase (NanoLuc)-based complementation assay that overcomes these issues and gives access to the profiling of ligands for their ability to induce GPCR interaction with individual G protein. In addition, the procedure is compatible with the settings of high-throughput screening. We applied this methodology to several prototypical class A receptors and their cognate G proteins. The D_2 receptor is a well-validated drug target in psychosis and Parkinson's diseases and is coupled to $G\alpha_{i/o}$ family. We used the D_2 receptor as a proof-of-concept to perform a screening of a library containing known drugs and active compounds against the D_2 receptor.

Results

The NanoLuc Binary Technology system can monitor Receptor-G protein interaction in living cells

In order to detect the real-time interaction between receptor and G proteins, we selected the NanoLuc Binary Technology (NanoBiT) that is based on NanoLuc, an engineered luciferase from the deep sea shrimp *Oplophorus gracilirostris* (23). We reasoned that the reported increased brightness of the enzyme would overcome sensitivity issues of other systems, such as firefly luciferase complementation, BRET or FRET. In addition, we

expected the small size of the NanoBiT partners (a small subunit (SmBiT, 11 AA)) and a larger subunit ((LgBiT, 158 AA), **Fig. 1A** (24)) could minimize perturbations of GPCR pharmacology that have been outlined with large fluorescent proteins (19).

As a proof of concept for such approach, we first selected three receptors coupled to the $G\alpha_{i/o}$ family: the long isoform of the Dopamine receptor subtype 2 (D_2) (25), the Histamine receptor H_3 (26) and the Succinate receptor (SUCNR1) (27). The SmBiT was attached to the C terminus of the receptors and the LgBiT was introduced in the loop connecting helices A and B of the $G\alpha_{i1}$ protein ($G\alpha_{i1}$-LgB^{91}) (**Fig. 1A**). The interaction between labeled receptor and $G\alpha_{i1}$-LgB^{91} was estimated by measuring the emitted light upon agonist stimulation. All the tested receptors induced a rapid increase of luminescent signal upon stimulation (**Fig. 2, A to C**). However, the amplitude and stability of the obtained signal was relatively weak. We reasoned that the transient nature of the GPCR-G protein interaction could, to some extent, explain the low level of the observed signal. SmBiT has been optimized to have a low affinity for LgBiT (K_D = 190 µM (24)) compared to the native sequence (natural peptide or NP, K_D =0.9 µM (24)) in order to minimize the perturbation of the physiological interaction induced by the presence of complementing partners (24). A third peptide with high affinity (HiBiT, K_D=0.7 nM (24)) has also been described (**Fig. 1B**). In order to improve the signal-to-noise ratio of our assay, we hypothesized that the affinity of the small complementing peptide could be a critical parameter for the sensitivity of the detection system. Therefore, we tested the other two peptides, NP and HiBiT fused at the same location than SmBiT on the test receptors. For the three receptors, the amplitude and the stability of the signal were markedly increased when NP was used as the complementation peptide (**Fig. 2, D-F**). For both these peptides, the signal remained stable for at least 10 minutes (**Fig. 2, D to I**), which is consistent with the literature describing real-time GPCR-G protein interaction (28, 29).

Nanoluc complementation is completely reversible when natural peptide or SmBiT are fused to the receptor

Next, we envisaged the possibility that the use of complementing partners with different affinities for each other could completely distort the GPCR-G protein interaction and increase the risk of detecting non-pharmacological interactions and the accumulation of irreversible receptor-G protein complexes. Thus, we tested the reversibility of the complementation between the three small peptides (SmBiT, NP and HiBiT) and LgBiT in our system. Following stimulation with dopamine, a competitive D_2 receptor antagonist (Sulpiride) was injected. For the D_2 receptor fused with the SmBiT, a rapid decrease of the NanoLuc signal that reached basal level was observed upon antagonist addition (**Fig. 3A**). When the experiment was repeated with the constructs containing NP, a similar pattern was observed, although the signal-to-noise ratio was increased (**Fig. 3B**). When the HiBiT was used as a partner, the rate of dissociation was markedly decreased and the signal did not reach the basal level, suggesting an incomplete dissociation of the receptor-G protein complex (**Fig. 3C**). In light of such results we decided to select the system using the NP for further investigations.

The GPCR-G protein NanoLuc complementation can be applied to all G protein subtypes

We questioned the possibility of implementing the detection system to other G protein subtypes. Thus, we also expanded our detection system to other families of Gα proteins. We introduced the LgBiT in the loop connecting helices A and B of the other Gα proteins and tested the long isoform of $G\alpha_s$ with the β_2 adrenergic receptor (β_2AR) (**Fig. 4A**), $G\alpha_q$ and $G\alpha_{11}$ with the histamine receptor H_1 (H_1) (**Fig. 4B**) and $G\alpha_{12}$ and $G\alpha_{13}$ with the thromboxane A_2 receptor (TPα) (**Fig. 4C**). Upon stimulation with their respective ligands, a significant interaction between the receptor and the Gα subunit was recorded in cells co-transfected with β_2AR-NP/$G\alpha_s$-LgB^{113}, H_1-NP/$G\alpha_{q/11}$-$LgB^{97}$and TPα-NP/$G\alpha_{12/13}$-$LgB^{115/106}$ (**Fig. 4, A to C**). We then aimed at performing a complete profiling of G protein recruitment on a test receptor. We chose the well-characterized D_2 dopaminergic receptor as a model because it has been previously reported to couple differently to a diversity of G proteins from the $G\alpha_{i/o}$ family (30). We designed additional sensors for the other α subunits of the $G_{i/o}$ family. We inserted the LgBiT at the same topological location in $G\alpha_{i2}$ and $G\alpha_{i3}$. For $G\alpha_o$ (the "a" isoform), the initial constructs gave poor signal and after different rounds of optimization the LgBiT was placed just after the helix C and a deletion of the first 52 amino acids at

the N-terminal side ($G\alpha_o$-LgB^{143d}) was performed. We stimulated with dopamine HEK293 cells transiently transfected with D_2-NP and each of these Gα subunits fused with LgBit. Exposure of D_2-NP to its endogenous agonist, dopamine, induced a significant increase of luminescence signal when the receptor was co-expressed in HEK293 cells with either $G\alpha_{i1}$-LgB^{91}, $G\alpha_{i2}$-LgB^{91}, $G\alpha_{i3}$-LgB^{91} or $G\alpha_o$-LgB^{143d} but no activity when the D_2-NP was co-transfected with plasmids containing the validated constructs $G\alpha_s$-LgB^{113}, $G\alpha_q$-LgB^{97}, $G\alpha_{11}$-LgB^{97}, $G\alpha_{12}$-LgB^{115} or $G\alpha_{13}$-LgB^{106} (**Fig. 4D**). The interaction profile of the D_2 with Gα subunits that was obtained with the present system was consistent with the ones already described in the literature (31-33).

The GPCR-G protein NanoLuc complementation system is amenable to high-throughput screening

We further examined whether the GPCR-G protein NanoLuc complementation assay could be used for a high-throughput screening campaign. In order to transpose this assay to a screening protocol, we first optimized the $G\alpha_{i1}$ construct. The LgBiT was inserted at different locations of the Gα protein and tested in presence of D_2 receptor tagged with the NP (**Fig. S1A**). The LgBiT placed at the N terminus of $G\alpha_{i1}$ ($G\alpha_{i1}$-$LgB^{N\text{-}term}$) showed the strongest signal among all the constructs tested. Similar investigations on G_o confirmed that $G\alpha_o$-LgB^{143d} was the construct that gave the best signal-to-noise ratio (**Fig. S1B**). Next, we performed a flow cytometry experiment to determine the relative expression of the D_2-NP receptor at the membrane compared to the intracellular expression of $G\alpha_{i1}$-$LgB^{N\text{-}term}$ and $G\alpha_o$-LgB^{143d} in transiently transfected HEK293 cells. We observed similar expression of receptor and Gα protein constructs for the two conditions of transfection (**Fig. S2**). In order to obtain the higher signal-to-noise ratio, we tested different stoichiometry of D_2-NP and $G\alpha_{i1}$-$LgB^{N\text{-}term}$/ $G\alpha_o$-LgB^{143d} for the transfection. A ratio 1:1 of receptor and Gα subunit revealed a higher signal-to-noise ratio and was used for the subsequent experiments (**Fig. S3A**). Next, we reasoned that the Gβγ dimer could affect the amplitude of the signal obtained, as it has been shown for the RET-based systems (28). However, the coexpression of $G\alpha_o$-LgB^{143d} with its complementary $G\beta_1\gamma_2$ dimer did not modify the recruitment of $G\alpha_o$-LgB^{143d} to D_2-NP. Interestingly, the coexpression of D_2-NP and $G\alpha_{i1}$-$LgB^{N\text{-}term}$ with $G\beta_1\gamma_2$ subunits decreased the signal of NanoLuc complementation upon dopamine stimulation, while it did not affect the pEC_{50} (**Fig. S3B**). Thus, the detection of the interaction between NP-tagged GPCR and the LgBiT-tagged Gα subunit does not require the coexpression with the Gβγ dimer.

Concentration-response curves that were determined on the system revealed specific interaction between D_2-NP/$G\alpha_{i1}$-$LgB^{N\text{-}term}$ (EC_{50} = 2 nM) and D_2-NP/ $G\alpha_o$-LgB^{143d} (EC_{50} = 116 nM) in the presence of dopamine (**Fig. 5, A and B**). During the kinetic measurement of the D_2-NP/$G\alpha_{i1}$-$LgB^{N\text{-}term}$ or the D_2-NP/ $G\alpha_o$-LgB^{143d} interaction induced by dopamine stimulation, we observed an increase of signal that started to decrease immediately upon competitive antagonist (Sulpiride and Spiperone) addition (**Fig. 5, C and D**). The affinity of Sulpiride for the D_2-NP construct that was estimated with a Schild plot (**Fig. S4**) was not significantly different between the two assays and was consistent with the literature (34). We applied statistical methods to determine if this assay would be suitable for high-throughput screening. We calculated the Z' factor which includes the mean and the standard deviation of the positive and negative control (35). This factor reflects the dynamic range of the assay and its ability to detect active ligands. A Z' factor that is comprised between 0.5 and 1 is characterized by a large band separation between positive and negative signals and can be considered as an excellent assay for hit identification (35). When cells co-expressing D_2-NP with $G\alpha_{i1}$-$LgB^{N\text{-}term}$ or $G\alpha_o$-LgB^{143d} were stimulated with either dopamine or the vehicle in a 96 well-plate, the Z' factors were of 0.61 and 0.69, respectively (**Fig. 5, E and F**).

$G\alpha_{i1}$- and $G\alpha_o$-based screening of a SOSA library identifies D_2 agonists with distinct pharmacological profiles

To further validate our approach, we screened a SOSA library composed of 1200 known active compounds and drugs (Prestwick chemical library®) on D_2-NP expressing cells together with the $G\alpha_{i1}$- or $G\alpha_o$-based complementation constructs ($G\alpha_{i1}$-$LgB^{N\text{-}term}$ or $G\alpha_o$-LgB^{143d}) in parallel. Several agonists of the D_2 receptor were present in this library and we reasoned that they would serve as internal positive controls. We fixed the threshold for hit identification as the mean of the negative control plus three times the standard deviation. With this

criterion, we detected the six agonists (the D_2 agonists were listed according to the BJP/IUPHAR database http://www.guidetopharmacology.org) of the D_2 receptor present in the library (**Fig. 6, A and B**).

Next, we tested a wide range of agonist concentrations and compared the profile of each ligand with regard to dopamine that was defined as the reference ligand. Surprisingly, the pEC_{50} of dopamine was very different between the $G\alpha_{i1}$ and $G\alpha_o$-based interaction assay (**Table 1**; pEC_{50}: 8.64 ± 0.10 and 6.60 ± 0.05, respectively). We postulated that the difference between pEC_{50} could be the consequence of using different constructs. Actually, when we tested several constructs of $G\alpha_{i1}$-LgB, we also obtained a difference of pEC_{50} upon stimulation with dopamine (**Fig. S1**).

We compared all data obtained with both $G\alpha_{i1}$ and $G\alpha_o$ assays relatively to dopamine. We observed that the E_{max} of the D_2 agonists were similar when we measured the interaction between D_2-NP and $G\alpha_{i1}$-$LgB^{N\text{-}term}$ (**Table 1**). However, when cells expressing D_2-NP and $G\alpha_o$-LgB^{143d} were stimulated with apomorphine, piribedil and pramipexole, we detected lower efficacies compared to the one of dopamine as these compounds behaved as partial agonist for the initiation of interaction between D_2 and $G\alpha_o$. For example, piribedil exhibited a decrease of efficacy up to 3-fold in comparison to the one measured for dopamine (**Fig. 7, A and B**). Furthermore, although pergolide was more potent than dopamine as an inducer of both the D_2-NP/$G\alpha_{i1}$-$LgB^{N\text{-}term}$ and D_2-NP/$G\alpha_o$-LgB^{143d} interactions (**Table 1**; pEC_{50} of 10.13 ± 0.07 and 8.26 ± 0.07 for $G\alpha_{i1}$-$LgB^{N\text{-}term}$ and $G\alpha_o$-LgB^{143d}, respectively), we observed a lower efficacy for pergolide when measuring the interaction between D_2-NP and $G\alpha_o$-LgB^{143d}. Ropinirole-promoted D_2-NP/$G\alpha_{i1}$-$LgB^{N\text{-}term}$ and D_2-NP/$G\alpha_o$-LgB^{143d} interactions were both less potent compared to dopamine (**Fig. 7, A and B**). In addition, relatively to dopamine, ropinirole showed a higher potency for inducing the D_2-NP/$G\alpha_{i1}$-$LgB^{N\text{-}term}$ compared to the D_2-NP/$G\alpha_o$-LgB^{143d} interaction (**Table 1**; pEC_{50} of 8.23 ± 0.06 and 5.89 ± 0.08 for $G\alpha_{i1}$ and $G\alpha_o$, respectively).

For the different tested ligands, there were no marked differences (when compared with dopamine) between the pEC_{50} for D_2-NP and $G\alpha_{i1}$-$LgB^{N\text{-}term}$ or D_2-NP and $G\alpha_o$-LgB^{143d} interactions (**Fig. 7C**). However, when we considered the E_{max}, partial agonism was recorded when measuring the interaction between D_2-NP and $G\alpha_o$-LgB^{143d} (**Fig. 7D**). This difference in measured E_{max} was more notable for piribedil and pergolide (**Fig. 7D**). For each tested ligand, $\Delta\log(E_{max}/EC_{50})$ was determined for both Gα constructs using dopamine as the reference ligand, followed by the calculation of the bias factor as $\Delta\Delta\log(E_{max}/EC_{50})$ ($\Delta\log(E_{max}/EC_{50})_{G\alpha i1\ pathway}$ - $\Delta\log(E_{max}/EC_{50})_{G\alpha o\ pathway}$) according to previously described method (36). A bias factor of 0 corresponds to absence of bias (relative to the reference ligand, dopamine), whereas a bias factor of 1 would means a 10-fold preference for inducing the $G\alpha_{i1}$ interaction. In opposite, a negative value would mean a preference for the $G\alpha_o$-dependent pathway. A bias factor of -0.27 (± 0.03) (apomorphine), -0.12 (±0.18) (piribedil), 0.04 (± 0.17) (pramipexole), 0.41 (± 0.12) (ropinirole) and 0.09 (± 0.18) (pergolide) was computed for D_2 agonists, demonstrating a significant bias for ropinirole and apomorphine through $G\alpha_{i1}$-$LgB^{N\text{-}term}$ and $G\alpha_o$-LgB^{143d}, respectively (**Fig. 7E**). Thus, the GPCR-G protein NanoLuc complementation assay is able to identify biased agonists for certain G proteins.

Furthermore, we tested the possibility of an extension of this assay to detect β-arrestin recruitment. Thus, we also evaluated these five D_2 agonists using dopamine as reference ligand in a β-arrestin 2-based NanoLuc complementation assay. We observed a partial activity for apomorphine, piribedil, ropinirole and pergolide, while pramipexole was a full agonist for β-arrestin 2 recruitment (**Fig. S5**). This assay was based on the same D_2-NP construct and a β-arrestin 2 tagged with the LgBiT at the N terminus. Thereby, we were able to monitor G protein or β-arrestin interaction using the same readout.

Discussion

In this study, our first goal was to develop a simple, robust and sensitive GPCR assay for the real-time detection of receptor-G protein interaction that would be amenable to high throughput screening.

The protein-fragment complementation assays have been devised on the principle that two complimentary parts of a reporter protein fused to

two putative partners would be detectable upon interaction (37). These strategies have greatly advanced our understanding of cellular biology, molecular biology or pharmacology. However, although they have a relatively high signal-to-noise ratio, they have been so far less popular compared to RET to monitor GPCR-G protein interactions. The main reason for this is that the complementation may perturb the natural interaction between the partners under scrutiny because of a possible intrinsic affinity between the two split parts of the reporter proteins or the irreversibility of the complementation once formed. For example, systems based on fluorescent protein of the GFP family or some β-galactosidase are unable to dissociate once re-formed and accumulate in the system (38). The Firefly and Gaussia luciferases seem to have a reduced propensity to form stable complexes but the split parts have affinity for each other and once reformed they are not very bright (39, 40).

Recently, a novel protein fragment complementation based on a brighter and smaller luciferase called NanoLuc has been described (24) and was already applied in the GPCR field to detect receptor-arrestin association (24, 39, 41). NanoLuc-based complementation presents several advantages compared to other RET- or complementation-based techniques. First of all, the system does not suffer from sensitivity issues because the protein that detects the interaction is a brighter luciferase (42). A second improvement is the small size of both the reconstituted luciferase and one of the complementing partners (13 AA only for the SmBiT, 11 AA for NP) that minimizes the risk for artefacts that would be induced by the bulky nature of the system constituents (24). A third advantage that we noticed is the dynamic reversibility of the system, at least when the SmBiT and NP were used on H_3, D_2 and SUCNR1 (see **Fig. 3B** and **Fig. 5 C&D**). It is important to note that we did not formally demonstrate that the true dynamic of GPCR-G protein interaction was preserved. Therefore, we cannot exclude that the rate of association/dissociation is impaired as a consequence of the complementation. Notwithstanding, the basal affinity of the complementing partners could still promote artefactual constitutive activity by bringing the G protein and the receptor in close vicinity. However, this assay was not designed to study these dynamics but to detect active ligands for uncharacterized receptors. Thus, the possible increase of sensitivity may actually be seen as beneficial.

Several strategies have been successfully applied in the past to specifically monitor the G protein that interacted with or was activated by a receptor bound by an agonist. Historically, the first reported attempts used cellular backgrounds such as the yeast or insect cells that are devoid of most of the G proteins or GPCR present in human cell lines such as HEK293 (43). Another screening-compatible assay that is able to monitor the activation of each G protein individually has been described recently (33). This elegant approach detects the interaction of the activated G protein (Gβγ subunits) with GRK3 with a NanoBRET system and was applied to profile the coupling between receptors and a large set of G proteins (33). Our approach differs in several aspects compared to the one described by Masuho *et al.* First, some G proteins, such as $G\alpha_{12}$, were not detectable by their system (33). Secondly, although the sensitivity and signal-to-noise ratio is relatively high, the authors did not test the possibility of using that assay for a screening campaign. Thirdly, the nature of the activating receptor giving rise to the recorded signal is not identifiable in a system solely based on G protein activation. Thus, a screening campaign on an elusive receptor with the assay described by Masuho *et al.* would give a high rate of false positive ligands that are activating the endogenous GPCRs present on the cell surface. Another putative way to monitor single G protein interaction to a receptor was published during the preparation of this manuscript. Using G proteins truncated at their N-terminal parts that were initially developed to facilitate crystallographic studies (called mini G protein), Wan *et al.* reported that they could monitor G protein-GPCR interactions but did not challenge the assay in screening conditions (44). Interestingly, we applied independently a reduction of the size of G protein to optimize our $G\alpha_o$ construct, further validating this strategy to improve the performance of engineered G proteins. Actually, all the constructs we developed did not give the same results in terms of sensitivity or signal-to-noise ratio. It should be taken into account when implementing the system for other receptors that several constructs should be tested to identify the best ones. This aspect is of

particular importance for orphan receptors were the confidence in assay performance is critical if no positive control exists.

BRET and FRET approaches have been extensively used to monitor G protein recruitment. In principle, a donor (a luciferase in the case of BRET or fluorescent protein in the case of FRET) is fused to one of the interacting partners and an acceptor fused to another. In case of sufficient proximity (the donor and acceptor must be at a distance below 10Å with a correct orientation (19)), the fluorescence of the acceptor can be detected at a unique wavelength while the recorded emission of the donor will be reduced (19). Although these elegant RET approaches shed light on exquisite aspects of receptors pharmacology such as G protein-GPCR association/dissociation rate or G protein recruitment fingerprints for individual ligands (29, 45, 46). They were never applied to library screenings probably because of their limited sensitivity. In addition, it should be noted that for RET systems to work, the presence of Gβγ is required with precise stoichiometry, which further limit their broad use, especially for screening campaigns. Here, we have demonstrated that the NanoLuc complementation applied to Gα does not necessitate the co-transfection of additional Gβγ subunits (Fig. S3B). For the ease of comparison, we have listed in the table **2** the advantages and limitations of different methods available to monitor G protein-GPCR interactions in living cells.

The dopaminergic system is composed of 5 dopamine receptors (D_1 & D_5 principally coupled to $G\alpha_{s/olf}$ and $D_{2\text{-}4}$ coupled mainly to $G\alpha_{i/o}$ family) of which several are validated therapeutic target for debilitating conditions such as Parkinson's disease (PD) or psychotic disorders (30). PD is a degenerative disorder marked by tremor, rigidity and slowness of movement due to a decline of dopaminergic neurons in the substantia nigra (47). In general, the dopaminergic drugs used to treat PD aim at restoring dopamine signalling in affected areas such as the striatum, notably through the activation of the receptor from the D_2 family (48). The screening of a library of known drugs on the D_2 receptor with a novel GPCR-G protein interaction assay detected five D_2 receptor agonists that are currently in use to treat patient suffering from PD (49). The full concentration-response curves that we determined for D_2-$G\alpha_{i1}$ and -$G\alpha_o$ interaction showed that while there were no relative differences (compared to dopamine) with regard to the potencies of the agonists (except for ropinirole), their E_{max} displayed signs of partial agonism when recruiting $G\alpha_o$, in particular for piribedil and pergolide (see **Fig. 7** and **Table 1**). The bias factors that we calculated indicate a different pharmacological profile for the different compounds, especially Ropinirole and Apomorphine. At this stage, the demonstration that the partial agonism on $G\alpha_o$ is linked to the therapeutic effect would be premature but the assay presented here has the potential to facilitate the discovery of compounds with a more pronounced bias that could be evaluated for a beneficial therapeutic effect.

Furthermore, the difference in pharmacological profile we observed between $G\alpha_{i1}$ and $G\alpha_o$ with the NanoLuc complementation on D_2 agonist are consistent with the literature. Pioneering work by the Strange lab in insect cells demonstrated that different agonists had the ability to elicit different responses depending on the G protein subtype (32, 50). Using G protein mutants resistant to PTX treatment, Milligan and his team managed to evidence similar behaviour of the D_2 receptor toward different G proteins of the $G\alpha_{i/o}$ family (31).

The current approaches to detect biased agonism are focused on the differences between G proteins and arrestins, were the assays to detect pharmacological parameters are, respectively, the measurement of second messengers and a complementation assay between arrestin and the active receptor (51). However, we think that it would be preferable to estimate bias with a common strategy instead of different assays because the assays are an important source of artefacts when determining bias. We demonstrated here that the nanoluciferase complementation could also be applied to the estimation of the arrestin recruitment (**Fig. S5**). Thus, a NanoLuc complementation can be applied to a more robust analysis of biased agonism between arrestin (or any other intracellular partner, in theory) and a given G protein.

In conclusion, the present study describes the development and usefulness of a novel system for the detection of direct interactions between GPCR and single G protein. This assay has a dynamic range that is compatible with high throughput

screening. It opens new avenues for programs aiming at the identification in large libraries and optimization of original scaffolds characterized by biased agonism at the level of G protein subtype, a feature that would be impractical with current technologies. Exquisite pharmacological tools such as biased agonists selectively promoting the interaction of a receptor with a restricted set of G proteins should ease our understanding of the physiological rationale for multiple coupling and apparently redundant G proteins.

Experimental Procedures

Materials

Dopamine, isoproterenol, succinate, histamine, imetit, sulpiride, piribedil, pramipexole and pergolide were from Sigma-Aldrich (St. Louis, MO). Ropinirole and U46619 were from Santa Cruz (Dallas, Texas, USA); spiperone from Abcam (Cambridge, UK). Prestwick Chemical Library® was from Prestwick Chemical (Illkirch, France).

Plasmids

Human D_2 Long, $G\alpha_{i2}$ and $G\alpha_{i3}$ were amplified from human ORFeome (version 7.1, http://horfdb.dfci.harvard.edu/hv7/). Human H_1, H_3, TPα, $G\alpha_{oa}$ and $G\alpha_s$ were amplified from pcDNA3.1$^+$ coding for each protein (cDNA Resource Center, Bloomsburg, PA). SUCNR1 coding sequence was amplified from genomic DNA of human embryonic kidney 293 (HEK293) cells. β_2AR, $G\alpha_{i1}$, $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{12}$ and $G\alpha_{13}$ were amplified from complementary DNA (cDNA) of HEK293 cells mRNA. All receptors were cloned into the pcDNA3.1$^+$ (Invitrogen, Carlsbad, CA) after addition of the Flag epitope (DYKDDDDK) at the N terminus, preceded by the signal sequence KTIIALSYIFCLVFA (Guan et al., 1992) for D_2 and β_2AR. After cloning into the pcDNA3.1$^+$, the SmBiT (VTGYRLFEEIL), NP (GVTGWRLCERILA) and HiBiT (VSGWRLFKKIS) were added with a flexible linker (GNSGSSGGGGSGGGGSSG) in frame to the C terminus of the receptor by polymerase chain reaction (PCR). All G proteins were cloned into the pcDNA3.1$^+$ (Invitrogen, Carlsbad, CA) after addition of the HA (YPYDVPDYA) epitope at the N terminus. Then, EcoRV and SacII sites (except BamHI and SacII for $G\alpha_{12}$) were inserted by PCR between specific amino acid residues of the Gα protein in order to insert the LgBiT. The coding sequence of LgBiT (VFTLEDFVGDWEQTAAYNLDQVLEQGGVSS LLQNLAVSVTPIQRIVRSGENALKIDIHVIIPYE GLSADQMAQIEEVFKVVYPVDDHHFKVILPY GTLVIDGVTPNMLNYFGRPYEGIAVFDGKKIT VTGTLWNGNKIIDERLITPDGSMLFRVTINS) was amplified by PCR and inserted, flanked by a flexible linker (SGGGGS) and the respective restriction sites, into the Gα subunit sequence. The location of the LgBiT was topologically identical for each of the following Gα subunits: between residues 91 and 92 of $G\alpha_i$ ($G\alpha_{i1}$-LgB^{91}, $G\alpha_{i2}$-LgB^{91}, $G\alpha_{i3}$-LgB^{91}) and $G\alpha_{oa}$ ($G_{\alpha o}$-LgB^{91}), residues 97 and 98 of $G\alpha_{q/11}$ ($G\alpha_q$-LgB^{97} and $G\alpha_{11}$-LgB^{97}), residues 113 and 114 of $G\alpha_s$ ($G\alpha_s$-LgB^{113}), residues 115 and 116 of $G\alpha_{12}$ ($G\alpha_{12}$-LgB^{115}) or residues 106 and 107 of $G\alpha_{13}$ ($G\alpha_{13}$-LgB^{106}). The $G\alpha_{i1}$ constructs used for the screening has been obtained as followed: the LgBiT was added at the N terminus of $G\alpha_{i1}$ by cloning its coding sequence into the pNBe3 vector (Promega Corporation, Madison, WI, USA) with XhoI and SacI sites and a HA tag was added at the C terminus sequence by PCR ($G\alpha_{i1}$-LgB$^{N\text{-}term}$). The $G\alpha_{oa}$ construct used for the screening was obtained by insertion of the LgBiT, flanked by a flexible linker (SGGGGS) and EcoRV/SacII sites, between residues 143 and 144 of $G\alpha_{oa}$ with a HA tag at the N terminus cloned into the pcDNA3.1$^+$ (Invitrogen, Carlsbad, CA). Then, the deletion of the first 52 amino acids was performed using the Q5 Site-Directed Mutagenesis Kit (New England Biolabs, Massachusetts, USA) ($G\alpha_o$-LgB^{143d}). For the β-arrestin 2-based NanoLuc complementation assay, β-arrestin 2 was cloned into pNBe3 vector (Promega Corporation, Madison, WI, USA) with XhoI and EcoRI sites. All constructs were verified by sequencing.

Cell Culture and Transfection

HEK293 cells were from American Type Culture Collection (Manassas, VA) and grown in Dulbecco's modified Eagle medium (Lonza, Verviers, Belgium) containing 10% fetal bovine serum (International Medical Products, Brussels, Belgium), 1% penicillin/streptomycin (Lonza), and 1% L-glutamine (Lonza) at 37°C with 5% CO_2. At 80% confluency, cells were transfected with XtremeGene 9 (Promega Corporation, Madison, WI, USA) in a 3:1 (reagent:DNA) ratio according to the manufacturer's recommendations. A 1:1 ratio (GPCR:G alpha subunit) was used with a solution of plasmids diluted 1/10 with empty pcDNA3.1$^+$.

Measurement of GPCR-G protein interaction by NanoLuc complementation assay

Twenty-four hours after transfection of HEK293 cells with a 1:1 ratio of GPCR and Gα subunit plasmid solutions diluted 1/10 with empty pcDNA3.1$^+$, cells were detached from 20 or 55 cm^2 dishes with trypsin. After one wash with PBS, cells were resuspended into Hanks' balanced salt solution (HBSS; 120 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO4, 10 mM HEPES, pH 7.4) supplemented with the NanoLuc substrate (Promega Corporation, Madison, WI, USA), seeded into a white 96-well plate (50,000 cells/well) and incubated for 45 minutes at 37°C. Cells were stimulated by adding 1 µl of 100x ligand solutions and luminescence was recorded for several minutes depending on each experiment (Centro XS^3 LB960; Berthold Technologies). The same protocol was used for β-arrestin 2 recruitment after a transient transfection of receptor and β-arrestin 2-encoding plasmids diluted 1/10 with empty pcDNA3.1$^+$ into HEK293 cells in a 1:1 ratio.

To determine the Z' factor, cells co-transfected with D_2-NP and $G\alpha_{i1}$-LgB$^{N\text{-}term}$ or D_2-NP and $G\alpha_o$-LgB^{143d} were resuspended into HBSS supplemented with NanoLuc substrate and seeded in a white 96-well plate (50, 000 cells/well) at 37°C for 45 min. Half of the plate was stimulated with vehicle and the other half with dopamine 100x concentrated and the luminescence was recorded for 10 min. The screening of the Prestwick Chemical Library® was performed at 5 µM on HEK293 transiently transfected with the pair of D_2-NPand $G\alpha_{i1}$-LgB$^{N\text{-}term}$ or $G\alpha_o$-LgB^{143d} (1:1 ratio with a solution of plasmids diluted 1/10 with empty pcDNA3.1$^+$). The cells were detached in parallel for $G\alpha_{i1}$ and $G\alpha_o$, resuspended into HBSS supplemented with the NanoLuc substrate (Promega Corporation, Madison, WI, USA) and incubated for 45 min at 37°C. Then, the compounds of the library (dissolved in dimethyl sulfoxide) were added into D_2-$G\alpha_{i1}$ plate and luminescence was recorded for 10 min. After, the compounds of the same plate of the library were added to D_2-$G\alpha_o$ plate and luminescence was recorded for 10 min.

Data Analysis and Hit Selection Criteria

All data show a representative result from 3 independent experiments, except data from Fig. 4 which represent the mean ± S.D. of 3 independent experiments. Data were collected as RLU during 10 min after stimulation and the areas under the curve were calculated and then normalized to the vehicle, considered as 0% activity. Statistical significance of the NanoLuc activity in presence of ligand from basal activity was assessed using nonparametric, Mann-Whitney test (***, $P < 0{,}001$). Concentration-response curves were fitted to the four-parameter Hill equation using the least-squares method (GraphPad Prism, version 5.0 for Windows; GraphPad, La Jolla, CA). To combine the results obtained for all tested ligands, we considered the E_{max} of dopamine as equal to 100% and represented the activity of each ligand compared to the activity of dopamine.

The Z' factor was calculated as follows: $Z' = 1 - ((3\sigma_{c+} + 3\ \sigma_{c-})/|\mu_{c+} - \mu_{c-}|)$, where σ, represents the standard deviation; µ, the mean; c+, positive control; c- negative control. For the screening of the Prestwick Chemical Library® compounds were considered as hits when the ratio > (mean $ratio_{(VEH)}$ + 3 $S.D._{(VEH)}$). Ratio represents $RLU_{(compound)}/RLU_{(VEH)}$. Bias factor was calculated in two steps. First, we determined the $\Delta\log(E_{max}/EC_{50})$ for $G\alpha_{i1}$ and $G\alpha_o$ pathway which represents the difference between D_2 agonist values and dopamine values used as reference ligand obtained with the $G\alpha_{i1}$- and $G\alpha_o$-based assay. Then, the bias factor corresponding to $\Delta\Delta\log(E_{max}/EC_{50})$ was calculated as follows : $\Delta\log(E_{max}/EC_{50})_{G\alpha i1\ pathway} - \Delta\log(E_{max}/EC_{50})_{G\alpha o\ pathway}$.

Acknowledgements: This work was supported by the Fonds pour la Recherche Scientifique (F.R.S.-FNRS) Incentive grant for scientific research (F.4510.14), University of Liège (Fonds Spéciaux) and Léon Fredericq Foundation. J.H. and C.L. are F.R.S.-FNRS research associate and PhD fellow, respectively. N.D. is an FRIA PhD fellow. The authors thank the GIGA Imaging and Flow Cytometry Platform. The Prestwick chemical library® used for the screening was a generous gift from Dr. Jean-Claude Twizere, laboratory of Protein Signaling and Interactions, GIGA-Molecular Biology of Diseases, university of Liège. The authors acknowledge the technical assistance of Céline Piron and they wish to thank Dr. Andy Chevigné for fruitful discussions and critical reading of the manuscript.

Conflict of interest: The authors declare no competing interests

Author Contribution:

Participated in research design: Laschet, Dupuis, Hanson.
Conducted experiments: Laschet, Dupuis
Contributed new reagents or analytic tools: Laschet, Dupuis.
Performed data analysis: Laschet, Dupuis, Hanson.
Wrote or contributed to the writing of the manuscript: Laschet, Dupuis, Hanson.

References

1. Hauser, A. S., Attwood, M. M., Rask-Andersen, M., Schiöth, H. B., and Gloriam, D. E. (2017) Trends in GPCR drug discovery: new agents, targets and indications. *Nat Rev Drug Discov*. **16**, 829–842
2. Sriram, K., and Insel, P. A. (2018) G Protein-Coupled Receptors as Targets for Approved Drugs: How Many Targets and How Many Drugs? *Molecular Pharmacology*. **93**, 251–258
3. Davenport, A. P., Alexander, S. P. H., Sharman, J. L., Pawson, A. J., Benson, H. E., Monaghan, A. E., Liew, W. C., Mpamhanga, C. P., Bonner, T. I., Neubig, R. R., Pin, J.-P., Spedding, M., and Harmar, A. J. (2013) International Union of Basic and Clinical Pharmacology. LXXXVIII. G Protein-Coupled Receptor List: Recommendations for New Pairings with Cognate Ligands. *Pharmacol. Rev.* **65**, 967–986
4. Ngo, T., Kufareva, I., Coleman, J. L., Graham, R. M., Abagyan, R., and Smith, N. J. (2016) Identifying ligands at orphan GPCRs: current status using structure-based approaches. *Br J Pharmacol*. **173**, 2934–2951
5. Laschet, C., Dupuis, N., and Hanson, J. (2018) The G protein-coupled receptors deorphanization landscape. *Biochemical Pharmacology*. **153**, 62–74
6. Roth, B. L., and Kroeze, W. K. (2015) Integrated Approaches for Genome-wide Interrogation of the Druggable Non-olfactory G Protein-coupled Receptor Superfamily. *Journal of Biological Chemistry*. **290**, 19471–19477
7. Kenakin (2012) Biased signalling and allosteric machines: new vistas and challenges for drug discovery. *Br J Pharmacol*. **165**, 1659–1669
8. Oldham, W. M., and Hamm, H. E. (2008) Heterotrimeric G protein activation by G-protein-coupled receptors. *Nat Rev Mol Cell Biol*. **9**, 60–71
9. Wettschureck, N., Offermanns, S., and Offermanns, S. (2005) Mammalian G proteins and their cell type specific functions. *Physiol Rev*. **85**, 1159–1204
10. Rajagopal, S., and Shenoy, S. K. (2018) GPCR desensitization: Acute and prolonged phases. *Cell Signal*. **41**, 9–16
11. Milligan, G., and Kostenis, E. (2006) Heterotrimeric G-proteins: a short history. *Br J Pharmacol*. **147 Suppl 1**, S46–55
12. Williams, C. (2004) cAMP detection methods in HTS: selecting the best from the rest. *Nat Rev Drug Discov*. **3**, 125–135
13. Gilissen, J., Geubelle, P., Dupuis, N., Laschet, C., Pirotte, B., and Hanson, J. (2015) Forskolin-free cAMP assay for Gi-coupled receptors. *Biochemical Pharmacology*. **98**, 381–391
14. Ma, Q., Ye, L., Liu, H., Shi, Y., and Zhou, N. (2017) An overview of Ca2+ mobilization assays in GPCR drug discovery. *Expert opinion on drug discovery*. **12**, 511–523
15. Inoue, A., Ishiguro, J., Kitamura, H., Arima, N., Okutani, M., Shuto, A., Higashiyama, S., Ohwada, T., Arai, H., Makide, K., and Aoki, J. (2012) TGFα shedding assay: an accurate and versatile method for detecting GPCR activation. *Nat Methods*. **9**, 1021–1029
16. Milligan, G. (2003) Principles: Extending the utility of [S-35]GTP gamma S binding assays. *Trends Pharmacol Sci*. **24**, 87–90
17. Schröder, R., Janssen, N., Schmidt, J., Kebig, A., Merten, N., Hennen, S., Müller, A., Blättermann, S., Mohr-Andrä, M., Zahn, S., Wenzel, J., Smith, N. J., Gomeza, J., Drewke, C., Milligan, G., Mohr, K., and Kostenis, E. (2010) Deconvolution of complex G protein-coupled receptor signaling in live cells using dynamic mass redistribution measurements. *Nat Biotechnol*. **28**, 943–949
18. Kostenis, E., Waelbroeck, M., and Milligan, G. (2005) Techniques: promiscuous Galpha proteins in basic research and drug discovery. *Trends Pharmacol Sci*. **26**, 595–602
19. Lohse, M. J., Nuber, S., and Hoffmann, C. (2012) Fluorescence/bioluminescence resonance energy transfer techniques to study G-protein-coupled receptor activation and signaling. *Pharmacol. Rev.* **64**, 299–336
20. Boute, N., Jockers, R., and Issad, T. (2002) The use of resonance energy transfer in high-throughput screening: BRET versus FRET. *Trends Pharmacol Sci*. **23**, 351–354

21. Machleidt, T., Woodroofe, C. C., Schwinn, M. K., Méndez, J., Robers, M. B., Zimmerman, K., Otto, P., Daniels, D. L., Kirkland, T. A., and Wood, K. V. (2015) NanoBRET—A Novel BRET Platform for the Analysis of Protein–Protein Interactions. *ACS Chem Biol*. 10.1021/acschembio.5b00143
22. Hoffmann, C., Gaietta, G., Bünemann, M., Adams, S. R., Oberdorff-Maass, S., Behr, B., Vilardaga, J.-P., Tsien, R. Y., Ellisman, M. H., and Lohse, M. J. (2005) A FlAsH-based FRET approach to determine G protein-coupled receptor activation in living cells. *Nat Methods*. **2**, 171–176
23. Hall, M. P., Unch, J., Binkowski, B. F., Valley, M. P., Butler, B. L., Wood, M. G., Otto, P., Zimmerman, K., Vidugiris, G., Machleidt, T., Robers, M. B., Benink, H. A., Eggers, C. T., Slater, M. R., Meisenheimer, P. L., Klaubert, D. H., Fan, F., Encell, L. P., and Wood, K. V. (2012) Engineered Luciferase Reporter from a Deep Sea Shrimp Utilizing a Novel Imidazopyrazinone Substrate. *ACS Chem Biol*. **7**, 1848–1857
24. Dixon, A. S., Schwinn, M. K., Hall, M. P., Zimmerman, K., Otto, P., Lubben, T. H., Butler, B. L., Binkowski, B. F., Machleidt, T., Kirkland, T. A., Wood, M. G., Eggers, C. T., Encell, L. P., and Wood, K. V. (2016) NanoLuc Complementation Reporter Optimized for Accurate Measurement of Protein Interactions in Cells. *ACS Chem Biol*. **11**, 400–408
25. Beaulieu, J.-M., Espinoza, S., and Gainetdinov, R. R. (2015) Dopamine receptors – IUPHAR Review 13. *Br J Pharmacol*. **172**, 1–23
26. Panula, P., Chazot, P. L., Cowart, M., Gutzmer, R., Leurs, R., Liu, W. L. S., Stark, H., Thurmond, R. L., and Haas, H. L. (2015) International Union of Basic and Clinical Pharmacology. XCVIII. Histamine Receptors. - PubMed - NCBI. *Pharmacol. Rev.* **67**, 601–655
27. Gilissen, J., Jouret, F., Pirotte, B., and Hanson, J. (2016) Insight into SUCNR1 (GPR91) structure and function. *Pharmacol Ther*. **159**, 56–65
28. Galés, C., Rebois, R. V., Hogue, M., Trieu, P., Breit, A., Hébert, T. E., and Bouvier, M. (2005) Real-time monitoring of receptor and G-protein interactions in living cells. *Nat Methods*. **2**, 177–184
29. Hein, P., Frank, M., Hoffmann, C., Lohse, M. J., and Bünemann, M. (2005) Dynamics of receptor/G protein coupling in living cells. - PubMed - NCBI. *EMBO J*. **24**, 4106–4114
30. Beaulieu, J. M., and Gainetdinov, R. R. (2011) The Physiology, Signaling, and Pharmacology of Dopamine Receptors. *Pharmacological Reviews*. **63**, 182–217
31. Lane, J. R., Powney, B., Wise, A., Rees, S., and Milligan, G. (2007) Protean Agonism at the Dopamine D2 Receptor: (S)-3-(3-Hydroxyphenyl)-N-propylpiperidine Is an Agonist for Activation of Go1 but an Antagonist/Inverse Agonist for Gi1,Gi2, and Gi3. *Molecular Pharmacology*. **71**, 1349–1359
32. Gazi, L., Nickolls, S. A., and Strange, P. G. (2003) Functional coupling of the human dopamine D2 receptor with G alpha i1, G alpha i2, G alpha i3 and G alpha o G proteins: evidence for agonist regulation of G protein selectivity. *Br J Pharmacol*. **138**, 775–786
33. Masuho, I., Ostrovskaya, O., Kramer, G. M., Jones, C. D., Xie, K., and Martemyanov, K. A. (2015) Distinct profiles of functional discrimination among G proteins determine the actions of G protein-coupled receptors. *Science Signaling*. **8**, ra123–ra123
34. Freedman, S. B., Patel, S., Marwood, R., Emms, F., Seabrook, G. R., Knowles, M. R., and McAllister, G. (1994) Expression and pharmacological characterization of the human D3 dopamine receptor. *J Pharmacol Exp Ther*. **268**, 417–426
35. Zhang, J., Chung, T., and Oldenburg, K. (1999) A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. *J Biomol Screen*. **4**, 67–73
36. Kenakin, Kenakin, T., Watson, C., Muniz-Medina, V., Christopoulos, A., and Novick, S. (2012) A simple method for quantifying functional selectivity and agonist bias. *ACS Chem. Neurosci.* **3**, 193–203
37. Michnick, S. W., Ear, P. H., Manderson, E. N., Remy, I., and Stefan, E. (2007) Universal strategies in research and drug discovery based on protein-fragment complementation assays. *Nat Rev Drug Discov*. **6**, 569–582
38. Magliery, T. J., Wilson, C. G. M., Pan, W., Mishler, D., Ghosh, I., Hamilton, A. D., and Regan, L. (2005) Detecting Protein−Protein Interactions with a Green Fluorescent Protein Fragment

Reassembly Trap: Scope and Mechanism. *J Am Chem Soc*. **127**, 146–157
39. Dupuis, N., Laschet, C., Franssen, D., Szpakowska, M., Gilissen, J., Geubelle, P., Soni, A., Parent, A.-S., Pirotte, B., Chevigné, A., Twizere, J.-C., and Hanson, J. (2017) Activation of the Orphan G Protein-Coupled Receptor GPR27 by Surrogate Ligands Promotes β-Arrestin 2 Recruitment. *Molecular Pharmacology*. **91**, 595–608
40. Hattori, M., Tanaka, M., Takakura, H., Aoki, K., Miura, K., Anzai, T., and Ozawa, T. (2013) Analysis of temporal patterns of GPCR-β-arrestin interactions using split luciferase-fragment complementation. *Mol. BioSyst.* **9**, 957–964
41. Szpakowska, M., Nevins, A. M., Meyrath, M., Rhainds, D., D'huys, T., Guité-Vinet, F., Dupuis, N., Gauthier, P.-A., Counson, M., Kleist, A., St-Onge, G., Hanson, J., Schols, D., Volkman, B. F., Heveker, N., and Chevigné, A. (2018) Different contributions of chemokine N-terminal features attest to a different ligand binding mode and a bias towards activation of ACKR3/CXCR7 compared with CXCR4 and CXCR3. *Br J Pharmacol*. **175**, 1419–1438
42. England, C. G., Ehlerding, E. B., and Cai, W. (2016) NanoLuc: A Small Luciferase Is Brightening Up the Field of Bioluminescence. *Bioconjug Chem*. **27**, 1175–1187
43. Liu, R., Wong, W., and Ijzerman, A. P. (2016) Human G protein-coupled receptor studies in Saccharomyces cerevisiae. *Biochemical Pharmacology*. 10.1016/j.bcp.2016.02.010
44. Wan, Q., Okashah, N., Inoue, A., Nehmé, R., Carpenter, B., Tate, C. G., and Lambert, N. A. (2018) Mini G protein probes for active G protein-coupled receptors (GPCRs) in live cells. *Journal of Biological Chemistry*. **293**, 7466–7473
45. Galés, C., Van Durm, J. J. J., Schaak, S., Pontier, S., Percherancier, Y., Audet, M., Paris, H., and Bouvier, M. (2006) Probing the activation-promoted structural rearrangements in preassembled receptor|[ndash]|G protein complexes. *Nature Structural & Molecular Biology*. **13**, 778–786
46. Saulière, A., Bellot, M., Paris, H., Denis, C., Finana, F., Hansen, J. T., Altié, M.-F., Seguelas, M.-H., Pathak, A., Hansen, J. L., Sénard, J.-M., and Galés, C. (2012) Deciphering biased-agonism complexity reveals a new active AT1 receptor entity. *Nat Chem Biol*. **8**, 622–630
47. Obeso, J. A., Rodriguez-Oroz, M. C., Goetz, C. G., Marin, C., Kordower, J. H., Rodriguez, M., Hirsch, E. C., Farrer, M., Schapira, A. H. V., and Halliday, G. (2010) Missing pieces in the Parkinson's disease puzzle. *Nat Med*. **16**, 653–661
48. Oertel, W., and Schulz, J. B. (2016) Current and experimental treatments of Parkinson disease: A guide for neuroscientists. *J Neurochem*. **139 Suppl 1**, 325–337
49. De Keyser, J., De Backer, J. P., Wilczak, N., and Herroelen, L. (1995) Dopamine agonists used in the treatment of Parkinson's disease and their selectivity for the D1, D2, and D3 dopamine receptors in human striatum. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*. **19**, 1147–1154
50. Cordeaux, Y., Nickolls, S. A., Flood, L. A., Graber, S. G., and Strange, P. G. (2001) Agonist regulation of D(2) dopamine receptor/G protein interaction. Evidence for agonist selection of G protein subtype. *J Biol Chem*. **276**, 28667–28675
51. Winpenny, D., Clark, M., and Cawkill, D. (2016) Biased ligand quantification in drug discovery: from theory to high throughput screening to identify new biased μ opioid receptor agonists. *Br J Pharmacol*. **173**, 1393–1403
52. Yano, H., Cai, N. S., Javitch, J. A., and Ferré, S. (2018) Luciferase complementation based-detection of G-protein-coupled receptor activity. - PubMed - NCBI. *BioTechniques*. **65**, 9–14

Abbreviations
GPCR: G protein-coupled receptors
GRK: GPCR kinase
AC: Adenylate cyclase
cAMP: Cyclic adenosine monophosphate
DAG: Diacylglycerol
IP_3: Inositol triphosphate
GEF: Guanine nucleotide exchange factor
D_2: long isoform of the Dopamine receptor 2
NanoBiT: NanoLuciferase Binary Technology
BRET: Bioluminescence resonance energy transfer
FRET: Fluorescence resonance energy transfer
LgB, Large BiT
H_3: Histamine receptor 3
SUCNR1: Succinate receptor
NP: Natural peptide
NanoLuc: Nanoluciferase
β_2AR: β_2 adrenergic receptor
H_1: histamine receptor 1
TPα: Thromboxane A_2 receptor α
RLU: Relative luminescence units
SD: Standard deviation
SEM: Standard error of the mean
IP_1: Inositol monophosphate
TGF-α: Transforming growth factor α
RET: Resonance energy transfer
GFP: Green fluorescent protein
PD: Parkinson's disease
PTX: Pertussis toxin
RAMPS: Receptor activity-modifying proteins
RGS: Regulators of G-protein signalling
GASPs: GPCR-associated sorting proteins
PDZ: PSD95/Disc Large/Zona Occludens
SH3: Src homology 3
PCR: Polymerase chain reaction

Table 1. Agonist activity on $G\alpha_{i1}$ and $G\alpha_o$ interaction of D_2 ligands

Ligand	Gi1		Go		Bias factor
	pEC_{50} (Std Error)	**E_{max} (%)**	**pEC_{50} (Std Error)**	**E_{max} (%)**	**$\Delta\Delta\log(E_{max}/EC_{50})$ (Std Error)**
Dopamine	8.64 (0.10)	100	6.60 (0.05)	100	0 (0.12)
Apomorphine	8.25 (0.04)	103	6.62 (0.04)	63	-0.27 (0.03)
Piribedil	8.28 (0.04)	112	6.76 (0.14)	42	-0.12 (0.18)
Pramipexole	8.59 (0.07)	119	6.80 (0.09)	77	0.04 (0.17)
Ropinirole	8.23 (0.06)	106	5.89 (0.08)	73	0.41 (0.12)
Pergolide	10.13 (0.07)	100	8.26 (0.07)	54	0.09 (0.18)

Agonist-induced $G\alpha_{i1}$ or $G\alpha_o$ interaction with D_2. Efficacy (E_{max}) relative to the maximum effect of dopamine (= 100%). Bias factor was calculated as described in materials and methods section.

Table 2. Reported techniques for the real-time profiling of GPCR-G protein coupling

Technique	Design	Advantages	Limitations	Amenable to HTS	references
FRET	CFP and YFP variants	- Labelling possible on intra- and extracellular side - No perturbation of the natural interaction	- Excitation and emission cross-talk - High background (Autofluore-scence) - Large size of the fluorescent proteins (27 KDa) - Addition of Gβγ dimer	No	(19, 29)
BRET	RLuc and GFP variants	- No perturbation of the natural interaction - No excitation by an external light source - low background	- low signal-to-noise ratio - large size (27 kDa) - Addition of Gβγ dimer	Not determined	(19, 28)
NanoBRET	Gβγ-venus and GRK3-NanoLuc	- improved signal compared to BRET - greater light output - Medium size of NanoLuc (19kDa)	- no tagged-receptor or Gα subunit - Large size of the venus (27kDa) - not applicable to $G\alpha_{12}$	Yes	(33)
NanoBiT "mini G proteins"	SmBiT (1-11) and LgBiT (12-169)	- brighter than BRET methods - low background - Small size of the SmBiT (1.3 kDa) - Medium size of LgBiT (18 kDa)	- non ideal emission for in vivo applications - modified G proteins - perturbation of the natural interaction	Not determined	(24, 44)
NanoLuc comple-mentation	NLuc1 (1-97) and Nl-Luc2 (98-171)	- brighter than BRET methods - high signal-to-noise ratio - low background - Medium size of the complementing partners (~9.5 kDa each)	- non ideal emission for in vivo applications - perturbation of the natural interaction	Not determined	(52)
Modified NanoBiT	NP (1-13) and LgBiT (14-171)	- brighter than BRET methods - high signal-to-noise ratio - low background - Small size of the NP (1.5 kDa) - Medium size of LgBiT (18 kDa)	- non ideal emission for in vivo applications - perturbation of the natural interaction	Yes	This study

Figure Legends

Figure 1. NanoLuc complementation system. (A) Constructs for the NanoLuc complementation assay between a GPCR linked to SmBiT, NP or HiBiT peptides and a Gα subunit linked to LgBiT. Upon stimulation (yellow circle), the coupling of heterotrimeric G protein to the receptor triggers the exchange of a GDP (purple star) by a GTP (green star) followed by dissociation of Gα from Gβγ subunits. The interaction between GPCR and Gα subunit induces the formation of a complete NanoLuciferase and light emission in the presence of its substrate. (B) Amino acid sequence and K_D of the different small peptides (SmBiT, NP and HiBiT). Red amino acids represent mutated amino acids compared to NP.

Figure 2. NanoLuc complementation measurement of interaction between $G\alpha_{i1}$ and GPCRs linked to SmBiT, NP or HiBiT peptides in living cells. NanoLuc activity kinetics measured in HEK293 cells coexpessing $G\alpha_{i1}$-LgB^{91} and GPCRs linked to three different small peptides indicated in each panel. (A, B and C) D_2, H_3 and SUCNR1 linked to SmBiT; (D, E and F) D_2, H_3 and SUCNR1 linked to NP; (G, H and I) D_2, H_3 and SUCNR1 linked to HiBiT, before and after injection of their respective ligand (dopamine 1 µM, imetit 100 nM and succinate 1 mM). Results are expressed as the normalization of the NanoLuc signal in presence of agonist to the signal in absence of agonist. Data are representative of the mean ± S.E.M. of at least 3 independent experiments.

Figure 3. Reversibility of the SmBiT, NP and HiBiT peptides. NanoLuc activity was measured in HEK293 cells coexpessing $G\alpha_{i1}$-LgB^{91} and D_2 linked to SmBiT (A), NP (B) or HiBiT (C), before and after stimulation with the receptor ligand dopamine (1 µM). Twenty minutes after addition of agonist, a selective-receptor antagonist sulpiride (10 µM) was injected. Data represent stimulated cells normalized to non-stimulated cells. Data are representative of the mean ± S.E.M. of at least 3 independent experiments.

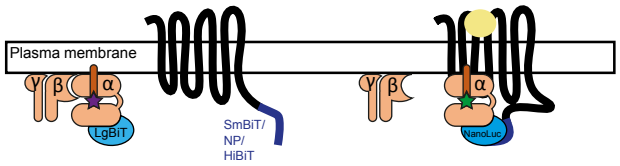
Figure 4. NanoLuc complementation measurement of GPCR and G protein interactions in living cells. (A-D) NanoLuc activity was measured in HEK293 cells coexpressing G alpha subunit linked to LgBiT and GPCR linked to NP as indicated in each panel. Cells were stimulated with vehicle or with their respective agonist (isoproterenol, histamine, dopamine 10 µM; U46619 10 nM) and the results are expressed as the normalization of agonist-treated cells to untreated cells. Data represent the mean ± S.D. of three independent experiments performed in triplicate. Statistical significance between stimulated and unstimulated cells was assessed using a nonparametric Mann-Whitney test (***, $P < 0.001$).

Figure 5. Screening compatible NanoLuc complementation assay for GPCR and G protein interactions. HEK293 cells coexpressing D_2-NP/$G\alpha_{i1}$-$LgB^{N\text{-}term}$ or $G\alpha_o$-LgB^{143d}. (A-B) Concentration-response curves of dopamine. (C-D) Kinetic measurements of NanoLuc activity during stimulation with dopamine and after addition of two different antagonists (sulpiride and spiperone). (E-F) Assay performance (Z' factor determination) for D_2 untreated and treated with dopamine (10 µM) on transiently cotransfected cells (n = 48). Data are representative of at least 3 independent experiments.

Figure 6. Screening of Prestwick chemical library® on D_2-$G\alpha_{i1}$ and D_2-$G\alpha_o$. (A-B) 1200 compounds were tested at the same concentration (5 µM) on cells coexpressing D_2-NP/$G\alpha_{i1}$-$LgB^{N\text{-}term}$ or $G\alpha_o$-LgB^{143d}. Results are expressed as the ratio over vehicle (DMSO)-treated (compounds) cells.

Figure 7. Pharmacological characterization of D_2 agonists on $G\alpha_{i1}$ and $G\alpha_o$ coupling. (A-B) Concentration-response curves of five D_2 agonists on $G\alpha_{i1}$ and $G\alpha_o$ interaction. E_{max} is expressed as the percentage of dopamine maximal activity. Estimation of pEC_{50} values (± S.D.) for each ligand and agonist efficacy measurements relative to dopamine are provided in table 1. Correlation between the pEC_{50} (± S.D.) (C) and Emax (± S.D.) (D) values determined in $G\alpha_{i1}$ and $G\alpha_o$ assays. (E) Bias factors [$\Delta\Delta\log(E_{max}/EC_{50})$] (± S.D.) calculated for tested D_2 agonists; positive values indicate bias for $G\alpha_{i1}$- over $G\alpha_o$-dependent pathway, while negative values indicate bias for $G\alpha_o$-dependent pathway. Unpaired Student's t tests were performed on the bias factors to determine the significance of ligand biases between $G\alpha_{i1}$ and $G\alpha_o$ pathways. Data points are representative of at least 3 independent experiments performed in triplicate.

A



B

Peptide	Sequence												K_D(M)
SmBiT	-	V	T	G	**Y**	R	L	**F**	E	**E**	I	L	-	1.9×10^{-4}
Natural peptide	G	V	T	G	W	R	L	C	E	R	I	L	A	0.9×10^{-6}
HiBiT	-	V	**S**	G	W	R	L	**F**	**K**	**K**	I	**S**	-	0.7×10^{-9}

Figure 1

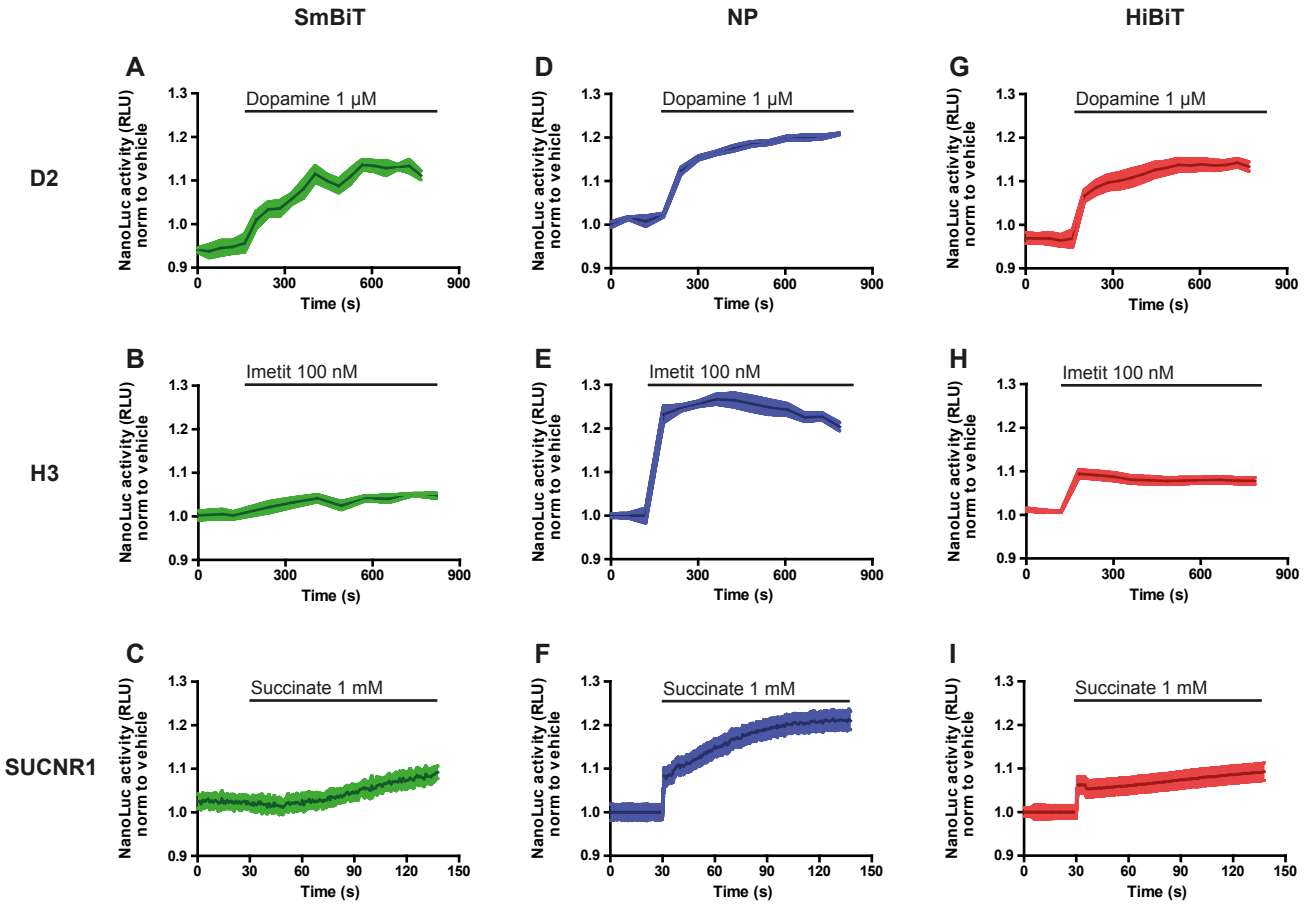

SmBiT
NP
HiBiT
D2
H3
SUCNR1
A
B
C
D
E
F
G
H
I
Dopamine 1 µM
Imetit 100 nM
Succinate 1 mM
NanoLuc activity (RLU) norm to vehicle
Time (s)

Figure 2

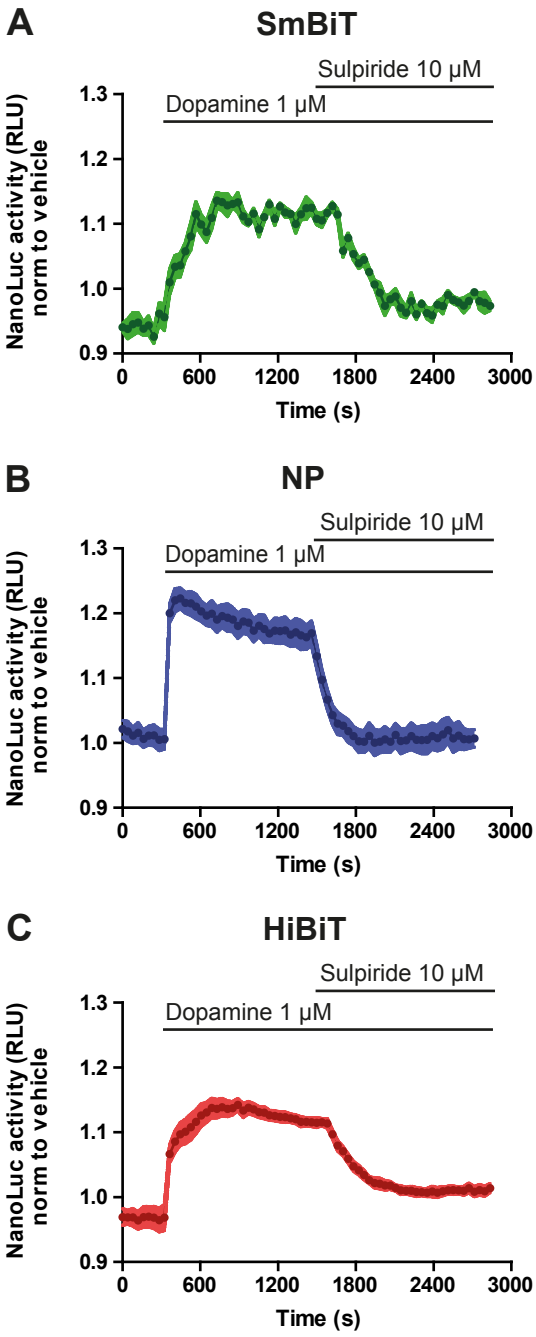

A
SmBiT
Sulpiride 10 µM
Dopamine 1 µM
NanoLuc activity (RLU)
norm to vehicle
1.3
1.2
1.1
1.0
0.9
0
600
1200
1800
2400
3000
Time (s)
B
NP
Sulpiride 10 µM
Dopamine 1 µM
NanoLuc activity (RLU)
norm to vehicle
1.3
1.2
1.1
1.0
0.9
0
600
1200
1800
2400
3000
Time (s)
C
HiBiT
Sulpiride 10 µM
Dopamine 1 µM
NanoLuc activity (RLU)
norm to vehicle
1.3
1.2
1.1
1.0
0.9
0
600
1200
1800
2400
3000
Time (s)

Figure 3

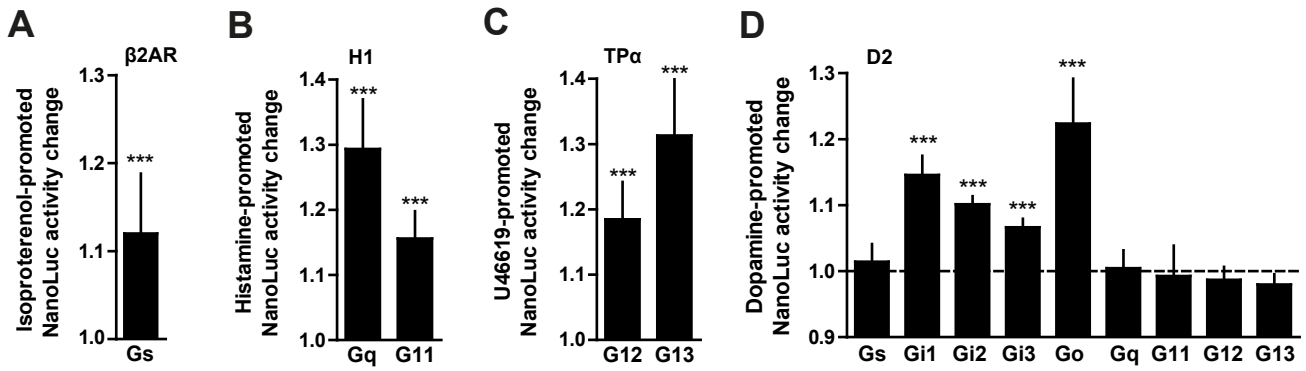

A
β2AR
Isoproterenol-promoted NanoLuc activity change
1.3
1.2
1.1
1.0
***
Gs
B
H1
Histamine-promoted NanoLuc activity change
1.4
1.3
1.2
1.1
1.0
***
***
Gq
G11
C
TPα
U46619-promoted NanoLuc activity change
1.4
1.3
1.2
1.1
1.0
***
***
G12
G13
D
D2
Dopamine-promoted NanoLuc activity change
1.3
1.2
1.1
1.0
0.9
***
***
***
***
Gs
Gi1
Gi2
Gi3
Go
Gq
G11
G12
G13

Figure 4

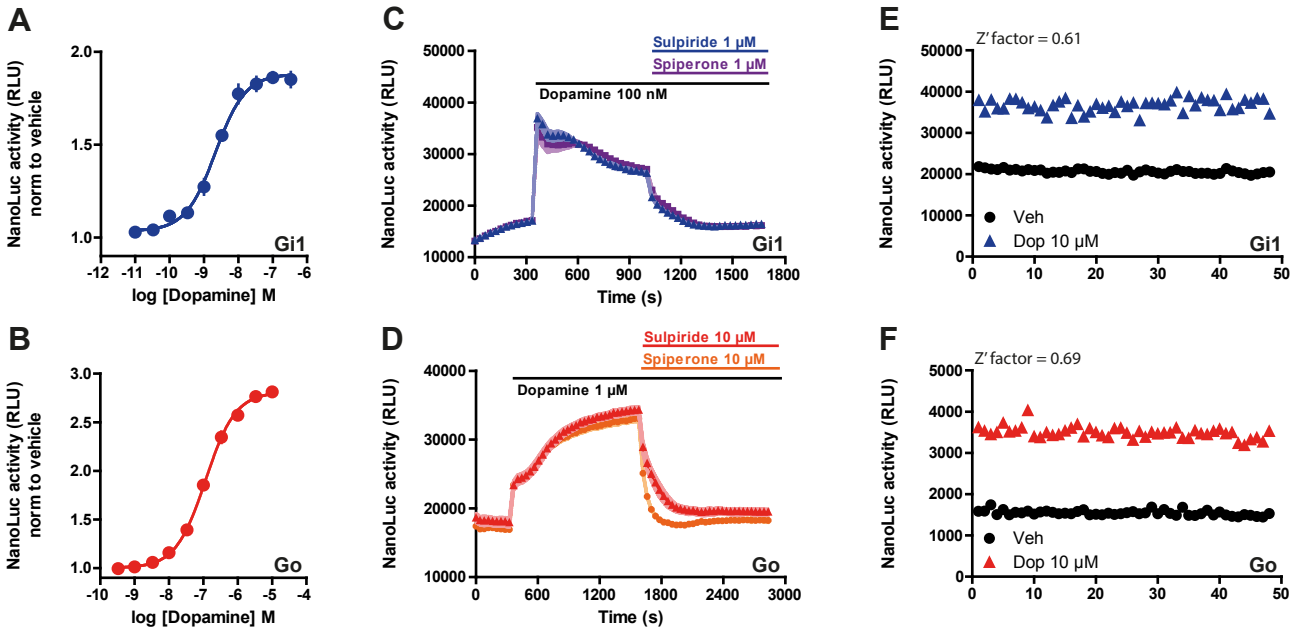

A
NanoLuc activity (RLU) norm to vehicle
2.0
1.5
1.0
-12 -11 -10 -9 -8 -7 -6
log [Dopamine] M
Gi1
C
NanoLuc activity (RLU)
50000
40000
30000
20000
10000
Sulpiride 1 µM
Spiperone 1 µM
Dopamine 100 nM
0 300 600 900 1200 1500 1800
Time (s)
Gi1
E
NanoLuc activity (RLU)
Z' factor = 0.61
50000
40000
30000
20000
10000
0
Veh
Dop 10 µM
0 10 20 30 40 50
Gi1
B
NanoLuc activity (RLU) norm to vehicle
3.0
2.5
2.0
1.5
1.0
-10 -9 -8 -7 -6 -5 -4
log [Dopamine] M
Go
D
NanoLuc activity (RLU)
40000
30000
20000
10000
Sulpiride 10 µM
Spiperone 10 µM
Dopamine 1 µM
0 600 1200 1800 2400 3000
Time (s)
Go
F
NanoLuc activity (RLU)
Z' factor = 0.69
5000
4000
3000
2000
1000
0
Veh
Dop 10 µM
0 10 20 30 40 50
Go

Figure 5

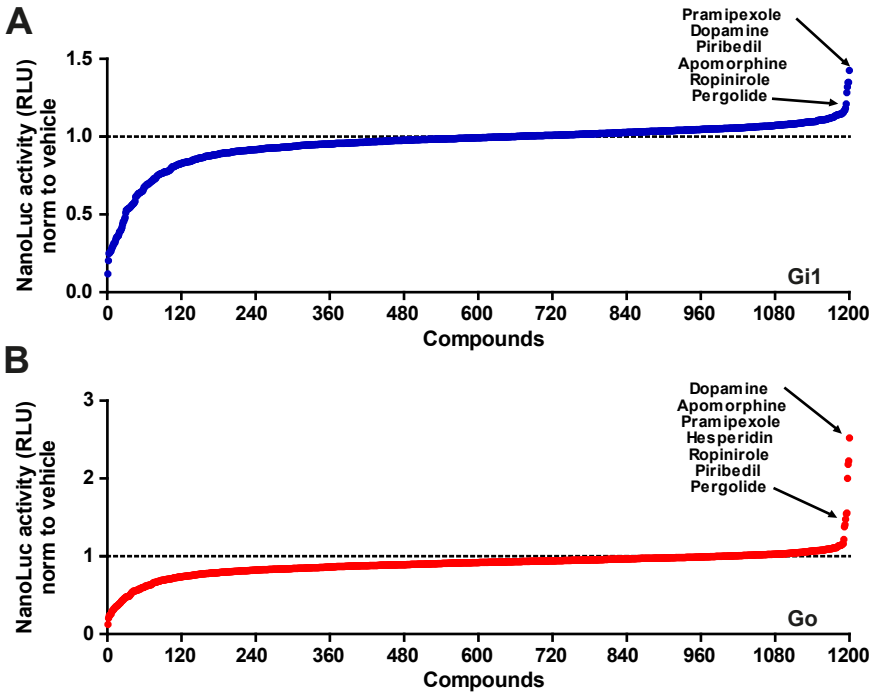

A
Pramipexole
Dopamine
Piribedil
Apomorphine
Ropinirole
Pergolide
NanoLuc activity (RLU)
norm to vehicle
1.5
1.0
0.5
0.0
Gi1
0
120
240
360
480
600
720
840
960
1080
1200
Compounds
B
Dopamine
Apomorphine
Pramipexole
Hesperidin
Ropinirole
Piribedil
Pergolide
NanoLuc activity (RLU)
norm to vehicle
3
2
1
0
Go
0
120
240
360
480
600
720
840
960
1080
1200
Compounds

Figure 6

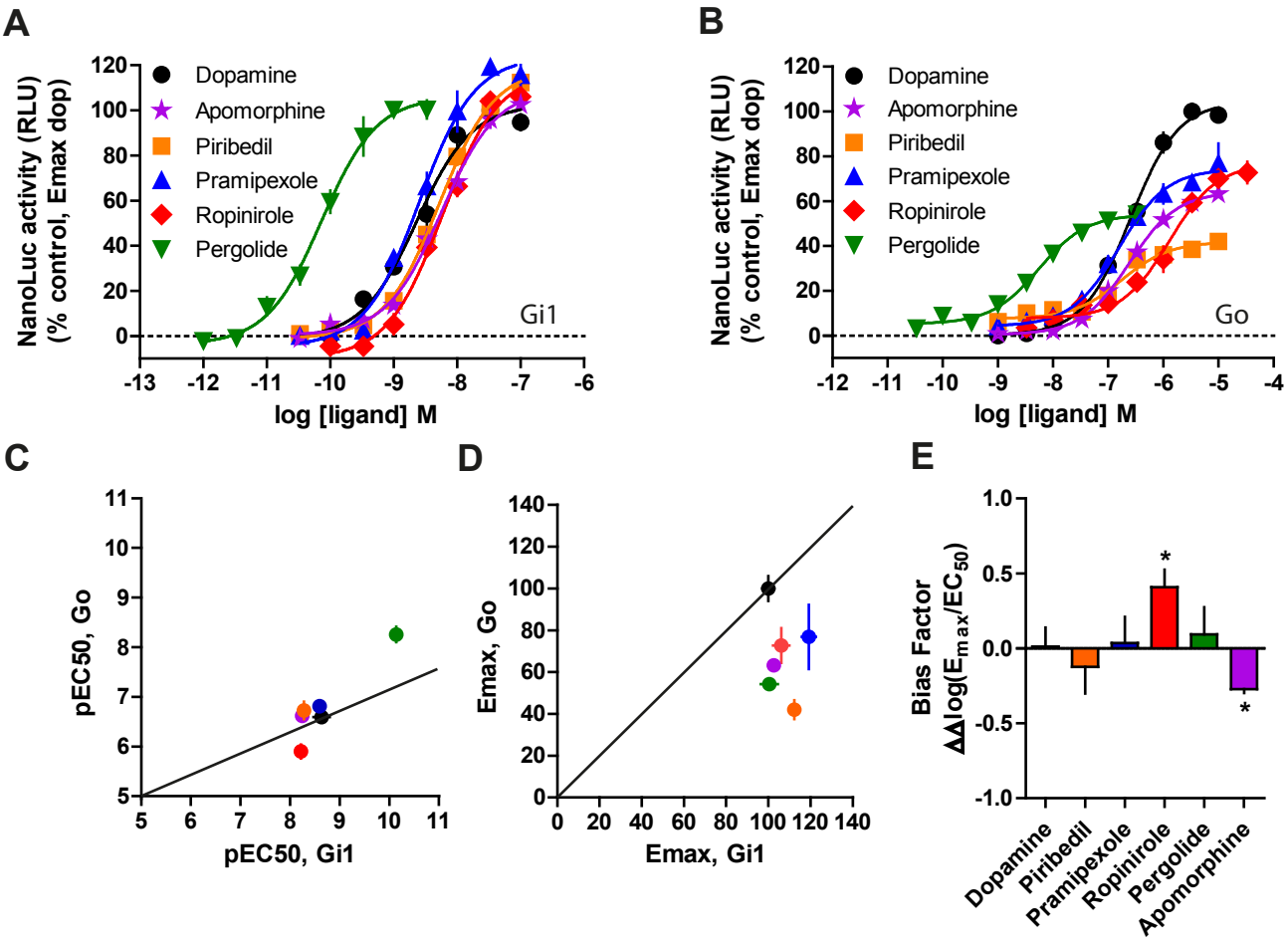

A
B
C
D
E
NanoLuc activity (RLU)
(% control, Emax dop)
Dopamine
Apomorphine
Piribedil
Pramipexole
Ropinirole
Pergolide
Gi1
Go
log [ligand] M
pEC50, Go
pEC50, Gi1
Emax, Go
Emax, Gi1
Bias Factor
ΔΔlog(Emax/EC50)
Dopamine
Piribedil
Pramipexole
Ropinirole
Pergolide
Apomorphine

Figure 7

A dynamic and screening-compatible nanoluciferase-based complementation assay enables profiling of individual GPCR-G protein interactions

Céline Laschet, Nadine Dupuis and Julien Hanson

J. Biol. Chem. published online December 28, 2018
