Antagonism of dopamine D2 receptor/β-arrestin 2 interaction is a common property of clinically effective antipsychotics

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Since the unexpected discovery of the antipsychotic activity of chlorpromazine, a variety of therapeutic agents have been developed for the treatment of schizophrenia. Despite differences in their activities at various neurotransmitter systems, all clinically effective antipsycho-

bic receptors (D2R), D2R mediate their physiological effects via both G protein-dependent and independent (β-arrestin 2-dependent) signaling, but the role of these D2R-mediated signaling events in the actions of antipsychotics remains unclear. We demonstrate here that while different classes of antipsychotics have complex pharmacological profiles at G protein-dependent D2R long isoform (D2LR) signaling, they share the common property of antagonizing dopamine-mediated interaction of D2R with β-arrestin 2. Using two cellular assays based on a bioluminescence resonance energy transfer (BRET) approach, we demonstrate that a series of antipsychotics including haloperidol, clozapine, aripiprazole, chlorpromazine, quetiapine, olanzapine, risperidone, and ziprasidone all potently antagonize the β-arrestin 2 recruitment to D2LR induced by quinpirole. However, these antipsychotics have various effects on D2R-mediated Gi/o protein activation ranging from inverse to partial agonists and antagonists with highly variable efficacies and potencies at quinpirole-induced cAMP inhibition. These results suggest that the different classes of clinically effective antipsychotics share a common molecular mechanism involving inhibition of D2LR/β-arrestin 2 mediated signaling. Thus, selective targeting of D2R/β-arrestin 2 interaction and related signaling pathways may provide new opportunities for antipsychotic development.

BRET | schizophrenia | signaling | functional selectivity

While the etiology of schizophrenia, a devastating chronic mental illness affecting 1–2% of human population, remains unclear, many of its symptoms can be attenuated by treatment with various classes of antipsychotic drugs. In fact, since the discovery of chlorpromazine in the 1950s and its antipsychotic properties, a large number of antipsychotics have been developed but their clinical efficacy and side-effect profiles could still be improved significantly. Generally, these compounds can be divided in 3 different categories: conventional (typical) antipsychotics, atypical antipsychotics, and the recently-introduced dopamine receptor partial agonist antipsychotics (dopamine-serotonin system stabilizers) (1). The second generation of antipsychotics (atypical and compounds with partial agonist activity at dopaminergic receptors) affect other neurotransmitter systems in addition to dopamine and offer advantages over the typical antipsychotics by targeting multiple schizophrenia symptoms and having less potential to induce extrapyramidal side effects or increase serum prolactin secretion (2). Nevertheless, the potential for other side effects still exist with these compounds mostly due to their off-target actions (3, 4).

While having complex pharmacological actions at several neurotransmitter systems, all antipsychotics principally act, albeit with different potency, on the dopamine (DA) system. It has been demonstrated that clinical efficacy of essentially all antipsychotic drugs (including traditional and newer antipsychotics) is directly correlated with dopamine D2 receptor (D2R) binding affinity and their capacity to antagonize this receptor (5, 6). It is commonly believed that the D2R, which belongs to the G protein-coupled receptor (GPCR) family, mediates the major part of its signaling and functions by coupling to G_{i/o} proteins to negatively regulate cAMP production. Thus, studies aimed at assessing the efficacy of antipsychotics on D2R signaling have classically been mainly concerned with measuring G_{i/o}-mediated inhibition of cAMP. However, recent evidence suggests that GPCRs may mediate their physiological functions by engaging signaling pathways via G protein-independent mechanisms (7). Upon stimulation, D2R, like other GPCRs, are regulated by processes leading to phosphorylation of the receptor by G protein-coupled receptor kinases (GRKs), uncoupling of the receptor from G protein activation, recruitment of the multifunctional scaffolding molecules, β-arrestins, and receptor internalization. Recent evidences indicate that β-arrestins, despite their critical involvement in regulating the GPCR’s desensitization and trafficking, can also be positive mediators for signaling, leading to formation of intracellular complexes of signaling molecules (7). In particular, D2R have been recently shown to engage the Akt/Glycogen Synthase Kinase 3 (GSK-3) signaling pathway by a G protein–independent mechanism that involves a previously unidentified signaling complex comprised of β-arrestin 2, Akt, and the multimeric protein phosphatase PP2A (8, 9).

This signaling mode, which is involved in the expression of certain dopamine associated behaviors (8, 10) and can be disrupted by lithium salts (11) seems to be mostly regulated by postsynaptically expressed dopamine D2Rs (9, 12).

Several lines of evidence support an important role for Akt and its downstream target GSK-3 in schizophrenia (10, 11, 13–17). In fact, lower Akt protein levels concomitant with a higher level of active GSK-3 have been found in brains of schizophrenic patients (13) and a significant association of Akt1 haplotypes with schizophrenia has been reported (14). Further-

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more, several antipsychotics can affect GSK-3 phosphorylation in various in vitro and in vivo experimental models (13, 15–17) and human postmortem studies (13). Altogether, these observations raise the possibility that the D2R-dependent β-arrestin 2/Akt/GSK-3 signaling pathway might be involved in the actions of antipsychotics.

Here, by using newly developed BRET assays, we compared the effects of several commonly used and putative antipsychotics on the D2L-R activation-induced recruitment of β-arrestin 2 and the Gs-dependent cAMP inhibition. We observed that while different antipsychotics have various actions on Gs pathway, all of them potently block dopamine-dependent β-arrestin 2 recruitment to D2L-R. These BRET assays could provide a simple in vitro system for predicting the potential antipsychotic activity of new compounds.

Results

BRET Assay to Monitor D2L-R-Mediated Activation of Gi/o. Gi/o-mediated cAMP inhibition is the best-characterized response to D2R activation. To measure and compare the agonist and antagonist activity of antipsychotics on this “classical” D2R signaling, we used a BRET adapted version of the ICUE 2 (indicator of cAMP using Epac) CAMP biosensor developed previously for FRET (18). For BRET experiments, the Exchange Protein Activated by CAMP (EPAC) was tagged at the C terminus with the Renilla luciferase and at the N terminus with citrine, an improved version of yellow fluorescent protein (YFP) (19). Sensitivity of this BRET CAMP sensor has already been validated (19) and was further confirmed by direct comparison of cAMP measurements performed using Enzyme Immuno Assay in response to D2L-R activation (data not shown). Stimulation of HEK cells coexpressing both the biosensor and D2L-R with the adenylylcyclase (AC) activator, forskolin (25 μM), induced a robust and sustained increase in cAMP production (Fig. 1A). Using this system, activation of Gi/o protein-coupled D2L-R and inhibition of AC can be monitored as the D2R agonist quinpirole (1 μM) abolished cAMP formation induced by forskolin. The maximal AC inhibition occurred immediately after agonist stimulation and remained constant for at least 20 min in the presence of the agonist (Fig. 1A).

As shown in Fig. 1B, quinpirole potently inhibited forskolin-stimulated cAMP accumulation in living cells with an EC50 of 5.7 nM ± 0.76. However, under the same conditions, dopamine generated a biphasic dose-response curve [supporting information (SI) Fig. S1]. In fact, at low concentrations (up to 0.1 μM), dopamine decreased cAMP levels, while at higher concentrations an increase in cAMP production was observed. This biphasic response of dopamine resulted from endogenously expressed dopamine D1-like receptors (D1R) in HEK cells as pretreatment of HEK cells with SCH23390, a selective D1R antagonist, generated a monophasic dopamine dose-response of cAMP inhibition with an EC50 of 18 nM ± 0.85 (Fig. S1). Also,
SKF81297, a selective D1R agonist, showed a monophasic dose-dependent increase in cAMP production (data not shown). Thus, the use of the selective D2R agonist quinpirole, in the assay, avoids this confound.

BRET Assay to Monitor β-arrestin 2 Recruitment to Activated D2R.
Given the potential importance of the β-arrestin 2-mediated signaling pathway in the actions of dopamine in vivo (8), and since little is known about the effect of antipsychotics on this pathway, we adapted a BRET assay to monitor activity of the antipsychotics on D2R mediated β-arrestin 2 recruitment as a readout of β-arrestin 2 mediated signaling. The use of β-arrestin recruitment to activated receptors has been developed to follow activation of multiple GPCRs and even used as a high-throughput screening tool to identify ligands (20, 21).

Murine dopamine D2R and β-arrestin 2 were cloned from a striatum cDNA library and fused to their C terminus with Renilla Luciferase (Rluc), the BRET donor, or the yellow variant of EGFP (YFP), the BRET acceptor, respectively. As measured by competitive binding, addition of the Rluc to the C terminus of D2R did not modify the affinity of tested antipsychotics for D2R (data not shown). BRET titration assays between D2R and β-arrestin 2 were carried out to assess whether agonist-promoted BRET changes reflected active recruitment of β-arrestin 2 to the receptor. In both unstimulated and stimulated HEK cells, a hyperbolic curve was obtained, demonstrating the progressive occupancy of the BRET donor with increasing quantity of the acceptor (Fig. 2A) (22). Stimulation of D2R with either quinpirole or dopamine at 1 μM induced a reduction of the BRET50 (ratio of YFP/Rluc leading to 50% of the maximal BRET signal) (BRET50 = 12.38; 7.76, and 5.43 for unstimulated, quinpirole, and dopamine respectively) and an increase of the maximal BRET (BRETmax = 0.066; 0.12, and 0.13 for unstimulated, quinpirole, and dopamine respectively) compared to unstimulated cells, which is consistent with an active recruitment of the β-arrestin 2 to the receptor (Fig. 2A). The basal and agonist-mediated interaction of D2R with β-arrestin 2 are specific since in control experiments, using free YFP as BRET acceptor, only much weaker and linear bystander BRET signals were observed with increasing YFP concentrations, indicating the nonspecific nature of the BRET signal arising from the interaction of soluble YFP with D2R-Rluc (see Fig. S2). This bystander BRET signal observed between YFP and D2R-Rluc was unaffected by dopamine or quinpirole treatment as can be seen by maximal BRET signal and BRET50 values that were similar to unstimulated cells. We next determined the kinetic of β-arrestin 2 recruitment to D2R after agonist stimulation using “real-time” BRET measurements. In cells expressing only D2R-Rluc, as expected, quinpirole at 1 μM did not induce BRET variations compared to untreated (PBS) cells. However, in cells coexpressing D2R-Rluc and β-arrestin 2-YFP, quinpirole rapidly increased BRET levels, which were stable for at least 20 min (Fig. 2B). Similar results were obtained using dopamine as agonist (data not shown). By comparing the basal BRET levels obtained from cells expressing D2R-Rluc alone and cells coexpressing both the receptor and the β-arrestin 2-YFP, we obtained a net BRET value of 0.03, reflecting the basal interaction of β-arrestin 2 with D2R in unstimulated cells. Fig. 2C shows dose-response curves for dopamine and quinpirole for β-arrestin 2 recruitment to D2R in living cells with EC50 values of 29.2 nM ± 0.81 and 97 nM ± 0.92, respectively.

Intrinsic Activity of Different Classes of Antipsychotics on D2R-Induced Signaling.
Using the BRET assays described above, we next evaluated the effects of different classes of antipsychotics on D2R signaling. First, the intrinsic activity of several antipsychotic compounds (haloperidol, clozapine, aripiprazole, des-methylclozapine, chlorpromazine, quetiapine, olanzapine, risperidone, and ziprasidone) on D2R-induced G_i/o activation and D2R-mediated β-arrestin 2 recruitment was determined in HEK cells stably expressing the EPAC biosensor (Fig. 3A and Fig. S3). In this assay, all of the antipsychotics acted as inverse agonists on D2R, with exception of aripiprazole that induced a decrease of cAMP levels. This effect of aripiprazole is not surprising since this compound is known to be a partial agonist at D2R with respect to modulation of the cAMP pathways (23). The efficacy and pEC50 values obtained for these compounds are summarized in Table 1. Next, the intrinsic activity of the same antipsychotics on β-arrestin 2 recruitment to D2R was also assessed. HEK cells coexpressing D2R-Rluc and β-arrestin2-YFP were stimulated with the different doses of antipsychotics. As shown in Fig. 3B and Fig. S4), none of these compounds were able to induce β-arrestin 2 recruitment to D2R. Importantly, aripiprazole, which acts as a partial agonist on the cAMP pathway, had no activity on D2R in this assay and did not induce β-arrestin 2 translocation either (see below).

Antagonist Activity of Different Classes of Antipsychotics on D2R Signaling.
Using the same assays, we next addressed the antagonistic properties of the antipsychotics on both quinpirole in-
Table 1. Intrinsic and antagonist activity of different antipsychotics on dopamine D2 receptor on the G_i/o pathway and β-arrestin 2 recruitment

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Intrinsic activity for G_i/o pathway</th>
<th>Intrinsic activity for β-arrestin 2 pathway</th>
<th>Antagonist activity for G_i/o pathway</th>
<th>Antagonist activity for β-arrestin 2 pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pEC_{50} E_{max} (%)</td>
<td>pEC_{50} E_{max} (%)</td>
<td>K_{B} (nM) E_{max} (%)</td>
<td>K_{B} (nM) E_{max} (%)</td>
</tr>
<tr>
<td>Quinpirole</td>
<td>8.25 ± 0.12 66.2 ± 0.05</td>
<td>7.01 ± 0.04 100 ± 1.01</td>
<td>N.A. N.A.</td>
<td>N.A. N.A.</td>
</tr>
<tr>
<td>Haloperidol</td>
<td>7.32 ± 0.39 42.6 ± 4.5</td>
<td>N.A. N.A.</td>
<td>0.28 ± 0.16 104 ± 4.5</td>
<td>0.12 ± 0.03 83.7 ± 2.5</td>
</tr>
<tr>
<td>Clozapine</td>
<td>6.97 ± 0.52 56.3 ± 9.8</td>
<td>N.A. N.A.</td>
<td>&gt;10,000 N.A.</td>
<td>67.7 ± 14.0 85.7 ± 8.2</td>
</tr>
<tr>
<td>Aripiprazole</td>
<td>9.45 ± 0.24 49.0 ± 3.9</td>
<td>N.A. N.A.</td>
<td>0.12 ± 0.05 29.9 ± 5.6</td>
<td>1.07 ± 0.32 69.6 ± 2.8</td>
</tr>
<tr>
<td>Desmethylclozapine</td>
<td>6.53 ± 0.52 28.0 ± 11.7</td>
<td>N.A. N.A.</td>
<td>&gt;10,000 N.A.</td>
<td>186 ± 107 98.2 ± 32.5</td>
</tr>
<tr>
<td>Chlorpromazine</td>
<td>8.81 ± 0.41 28.6 ± 4.0</td>
<td>N.A. N.A.</td>
<td>0.72 ± 0.1 95.4 ± 5.1</td>
<td>5.04 ± 1.21 95.6 ± 6.2</td>
</tr>
<tr>
<td>Quetiapine</td>
<td>7.40 ± 0.40 28.0 ± 5.0</td>
<td>N.A. N.A.</td>
<td>&gt;10,000 N.A.</td>
<td>88.5 ± 8.8 84.6 ± 6.4</td>
</tr>
<tr>
<td>Olanzapine</td>
<td>8.60 ± 0.65 33.8 ± 5.6</td>
<td>N.A. N.A.</td>
<td>2.10 ± 0.6 105 ± 7.4</td>
<td>1.95 ± 0.28 83.8 ± 3.5</td>
</tr>
<tr>
<td>Risperidone</td>
<td>9.25 ± 0.35 16.4 ± 2.8</td>
<td>N.A. N.A.</td>
<td>1.08 ± 0.9 96.3 ± 7.0</td>
<td>0.02 ± 0.01 83.0 ± 3.0</td>
</tr>
<tr>
<td>Ziprasidone</td>
<td>10.0 ± 1.2 23.8 ± 6.1</td>
<td>N.A. N.A.</td>
<td>0.13 ± 0.04 88.7 ± 7.3</td>
<td>0.03 ± 0.02 69.3 ± 3.6</td>
</tr>
</tbody>
</table>

The values of K_B, representing the molecular affinity of the competitive antagonist, were determined using the equation: K_B = I_{50}/ [(2 + ([A]/EC_{50}^{p^{1/n}}))]^{1/n}, where the concentration of agonist is [A]; the concentration of agonist producing 50% maximal response is EC_{50}, and n is the Hill coefficient of the agonist dose-response curve (29). Abbreviation: N.A.: not applicable. For the intrinsic activity of the antipsychotics for G_i/o, efficacy is expressed in percent of forskolin effect. For the antagonistic activity of the antipsychotics on G_i/o pathway and β-arrestin 2 recruitment, efficacy is expressed in percent of quinpirole maximum effect (1 μM).

* pEC_{50} of agonist activity for G_i/o pathway.

Dose-response curves of the different antipsychotics on quinpirole-induced cAMP inhibition are shown in Fig. 4A and Fig. S5. The K_B and efficacy values of the compounds tested are presented in Table 1. Almost all of the compounds were able to block dose-dependently quinpirole effects but with markedly different potencies and efficacies (Table 1). Thus, with the exception of clozapine, desmethyleclozapine, and quetiapine that had low efficacies against 1 μM quinpirole, all of the other antipsychotics blocked the effects of quinpirole almost completely. However, when quinpirole was used at a lower concentration, 100 nM, clozapine, desmethyleclozapine, and quetiapine were able to completely abolish quinpirole effect dose-dependently (data not shown). Unlike the other antipsychotics, aripiprazole blocked quinpirole-induced β-arrestin 2 recruitment with maximum efficacy of only 30% likely due to its partial agonist activity on D2_R (see Fig. 3A and Table 1).

We next determined the antagonist activity of these antipsychotics on β-arrestin 2 recruitment induced by quinpirole. As typically shown in Fig. 4B (and Fig. S6), all antipsychotics were highly efficacious in blocking quinpirole induced β-arrestin 2 translocation to the D2_R. The different potencies of all drugs tested for inhibiting translocation are summarized in Table 1. Strikingly, aripiprazole, which acts as a partial agonist on the cAMP pathways but has no intrinsic agonistic activity on β-arrestin 2 recruitment, was able to fully antagonize quinpirole-induced β-arrestin 2 recruitment with a K_B of 1.07 nM ± 0.32 (see Fig. 3B). Similarly, with the exception of chlorpromazine and olanzapine, all other antipsychotics displayed potencies toward the β-arrestin 2 recruitment that were 3–150-fold higher than at the G protein-mediated pathway. To further confirm the results obtained by BRET assay, confocal microscopy in living cells was used to visualize the effect of haloperidol, clozapine, and aripiprazole on the β-arrestin 2 recruitment to D2_R. HEK cells coexpressing human D2R and YFP-tagged β-arrestin 2 were stimulated with quinpirole (Fig. 5 Upper Right) or the antipsychotics (Fig. 5, Lower Center) at 10 μM. After 30 min of treatment, only quinpirole induced formation of fluorescent puncta at the plasma membrane reflecting β-arrestin 2 translocation. In unstimulated cells, β-arrestin 2 tagged with YFP was distributed throughout the cytosol (Fig. 5, Upper Left). Pretreatment of cells for 30 min with the antipsychotics completely abolished β-arrestin 2 recruitment induced by quinpirole confirming the results obtained by BRET with the murine D2_R (Fig. 5, Lower Left, Center, and Right).

**Discussion**

Dysregulation of the dopamine system and its physiological actions through the D2-like receptors are believed to contribute to either the pathogenesis or manifestations of schizophrenia. Dopamine D2Rs are classical GPCRs and are coupled to G_i/o. However, D2R activation can also regulate the Akt-GSK3 pathway through a G protein independent mechanism involving the scaffolding protein β-arrestin 2 (8). Since the Akt-GSK3 signaling pathway has been suggested to be involved in schizophrenia (10, 11, 13–17, 24) and that all clinically effective antipsychotics act, at least in part, by interacting with D2R, we compared the potencies of antipsychotics with regards to blocking β-arrestin 2 recruitment to D2R versus their well studied effects on G_i/o pathway activation. At least in the striatum, the negative regulation of Akt by dopamine was shown to be mostly a postsynaptic phenomena regulated by D2_R (9). Since both the long and the short isoforms of D2R have different functions in vivo with D2_R acting mainly at postsynaptic sites (12), we investigated signaling pathways activated by D2_R. For this purpose, we adapted two BRET approaches that allowed us to monitor both the β-arrestin translocation to D2_R and the G_i/o protein activation.

Using these assays, we observed that all antipsychotics tested have no intrinsic activity with regards to D2_R induced β-arrestin 2 recruitment, while they all (with the exception of aripiprazole) act as inverse agonists at the G_i/o pathway. Assessment of the antagonist potencies of the antipsychotics on these two pathways showed a markedly lower K_B for the majority of the compounds to antagonize β-arrestin 2 recruitment compared to AC inhibition induced by quinpirole (see Table 1). Thus, there seems to be a clear difference between the potencies of antipsychotics to antagonize these two signaling paradigms allowing them to discriminate one pathway versus the other. Ultimately, it would be interesting to explore whether the efficacies of these compounds as antipsychotics and their relative liability for extrapyramidal side effects, correlate with their profile for either of these pathways. However, the observed differences in the activity of antipsychotics raise important questions regarding the molecular mechanism underlying the preferential antagonism of the arrestin pathway. Moreover, antipsychotics can also interact with and affect β-arrestin 2 recruitment to the presynaptic D2R.
short isoform (data not shown). Whether antipsychotics can selectively modulate different putative signaling modes of D2R and contribute to their in vivo effects may be interesting to examine.

While aripiprazole displays partial agonism at dopamine D2R coupling to Gi, our results clearly show that aripiprazole has no effect by itself on β-arrestin 2 recruitment to D2R while potently antagonizing β-arrestin 2 translocation induced by quinpirole or dopamine. Aripiprazole has been shown to lack the ability to induce internalization of the long form of D2R (25), a mechanism known to be β-arrestin dependent. The fact that aripiprazole at the same time exerts partial agonist activity with regards to cAMP production highlights the functional selectivity of aripiprazole at D2R for the Gi pathway. Our data provide support for the hypothesis that aripiprazole is a functionally selective agent (biased agonist) for D2R rather than simply a partial agonist (26). It is likely that D2R can exist in an ensemble of distinct conformations that could engage different signaling modalities in response to the binding of different ligands. In fact, such functional selectivity has been shown recently for different GPCRs such as the β2-adrenergic receptor, angiotensin II type 1 receptor (AT1), CCR7, CXCR4, and the μ-opioid receptor (for review see ref. 27). Furthermore, one leading hypothesis to explain the unique clinical properties of atypical antipsychotics such as clozapine, postulates the importance of non-dopaminergic actions, particularly at serotonin receptors. While this study did not assess directly the effects of these compounds on serotonin receptor functions, we found that all clinically effective antipsychotics block with high potency D2R/β-arrestin 2 recruitment. Thus, antagonism at the D2R/β-arrestin 2-dependent pathway rather than their actions on D2R/Gi pathway or other neurotransmitter systems could have better predictive value for their therapeutic efficacy.

In summary, we investigated the activity of antipsychotics on the different modalities of D2R signaling involving the Gi-mediated cAMP production is highly variable. Evaluation of drug activity on β-arrestin 2 recruitment to D2R may provide an effective approach for the development of future antipsychotics.

**Methods**

**Reagents.** All cell culture reagents and buffers were from Gibco and Sigma, and FBS from JRH Biosciences. Quinpirole, dopamine, isoproterenol, haloperidol, clozapine, chlorpromazine, and forskolin were purchased from Sigma. Collagen was from Roche. Aripiprazole, desmethylclozapine, olanzapine, quetiapine, risperidone, and ziprasidone were provided by H. Lundbeck A/S. Coelenterazine h was purchased from Promega.

**Cell Culture and Transfections.** Human embryonic kidney 293 cells (HEK293T; Gibco and Sigma) were maintained in Dulbecco’s Modified Eagle’s medium supplemented with 10% (vol/vol) of FBS, 2 mM L-glutamine and 0.05 mg/ml of Gentamicin at 37°C in a humidified atmosphere at 95% air and 5% CO2.
Transient transfections were performed 24 h after cell seeding using calcium phosphate protocol.

**Bioluminescence Resonance Energy Transfer Measurement.** For BRET assays phenol red free medium was removed from HEK293T cells and replaced by Phosphate Buffer Saline (PBS) containing calcium and magnesium and 0.003% (wt/vol) ascorbic acid.

For measurement of cAMP variation using the EPAC biosensor, the experiments and reads were performed at 37°C using Mithras LB 940 instrument (Berthold). The assay was started by adding 10 µl of the cell-permeant substrate specific for Renilla luciferase, coelenterazine h to the well to yield a final concentration of 5 µM. The agonist activity of the compounds was measured by adding those 5 min after the Fluc substrate, and forskolin was then added to the wells 5 min after agonists. Reads of the plates started 5 min after the addition of forskolin. When the assay was conducted to determine antagonist properties of compounds against quinpirole, the different compounds were added simultaneously 1 min after the coelenterazine h. To follow the β-arrestin 2 recruitment to the dopamine D2R receptor, the same protocols as above were used except that experiments and reads were performed at room temperature. Reads started 10 min after the addition of Fluc substrate and no forskolin was used.

BRET readings were collected using a Mithras LB940 instrument that allows the sequential integration of the signals detected in the 465 to 505 nm and 515 to 555 nm windows using filters with the appropriate band pass and by using MicroWin 2000 software.

For titration experiments, the acceptor/donor ratio was calculated as previously described (28).

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