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Recruitment of β -arrestin2 to the dopamine D2 receptor: Insights into anti-psychotic and anti-parkinsonian drug receptor signaling

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Abstract

Drugs acting at dopamine D2-like receptors play a pivotal role in the treatment of both schizophrenia and Parkinson's disease. Recent studies have demonstrated a role for G-protein independent D2 receptor signaling pathways acting through β -arrestin. In this study we describe the establishment of a Bioluminescence Resonance Energy Transfer (BRET) assay for measuring dopamine induced recruitment of human β -arrestin2 to the human dopamine D2 receptor. Dopamine, as well as the dopamine receptor agonists pramipexole and quinpirole, acted as full agonists in the assay as reflected by their ability to elicit marked concentration dependent increases in the BRET signal signifying β -arrestin2 recruitment to the D2 receptor. As expected from their effect on G-protein coupling and cAMP levels mediated through the D2 receptor RNPA, pergolide, apomorphine, ropinirole, bromocriptine, 3PPP, terguride, aripiprazole, SNPA all acted as partial agonists with decreasing efficacy in the BRET assay. In contrast, a wide selection of typical and atypical anti-psychotics was incapable of stimulating β -arrestin2 recruitment to the D2 receptor. Moreover, we observed that haloperidol, sertindole, olanzapine, clozapine and ziprasidone all fully inhibited the dopamine induced β -arrestin2 recruitment to D2 receptor (short variant) in a concentration dependent manner. We conclude that most anti-psychotics are incapable of stimulating β -arrestin2 recruitment to the dopamine D2 receptor, in accordance with their antagonistic properties at the level of G-protein coupling.

Keywords

Dopamine; β -Arrestin; Anti-psychotic; D2; DRD2; Signaling; Akt

1. Introduction

Dopamine signaling in the central nervous system is mediated by dopamine D1-like (D1 and D5) and D2-like (D2, D3 and D4) receptors. The dopamine receptors are G-protein coupled and in striatal medium spiny neurons (MSN), the D1 and D2 receptors control downstream signaling in response to dopamine via their opposing effects on cAMP production. Imbalances in dopamine signaling in the basal ganglia are believed to play an integral part in the disease manifestation of Parkinson's disease and schizophrenia. All approved anti-psychotic medicines act, with respect to G-protein coupling, either as antagonists, inverse agonists or low efficacious partial agonists at the D2-like receptors. Moreover, their therapeutic active dose correlates with the occupancy at the D2 receptor (Akam and Strange, 2004; Burstein et al., 2005; Richtand et al., 2007).

For a number of G-protein coupled receptors (GPCR), including the β -adrenergic and V2 vasopressin receptors, it has been demonstrated that several agonists and inverse agonists exhibit biased agonism as reflected in differential activation of downstream signaling pathways that are not proportional to the G-protein mediated signaling (Urban et al., 2007a). For the long transcriptional variant of dopamine D2 (D2_L) receptor, it has been shown that the agonists *S*(+)-10,11-dihydroxy-*N*-*n*-propylnoraporphine (SNPA), *R*(-)-10,11-dihydroxy-*N*-*n*-propylnoraporphine (RNPA) and dihydrexidine are as efficacious as the full agonist quinpirole in inhibition of cAMP production. However, both RNPA and SNPA do not activate G-protein coupled inwardly rectifying K⁺ (GIRK) channels and SNPA exhibits partial agonism towards D2_L mediated MAP kinase phosphorylation, whereas dihydrexidine shows partial agonism for GIRK channel activation (Gay et al., 2004).

The ubiquitously expressed non-visual β -arrestins are pivotal for regulation of desensitization and internalization of most GPCRs but have also been shown to act as scaffolds in signal transduction events independent of G-protein mediated signaling (Azzi et al., 2003; Violin and Lefkowitz, 2007). For example, the inverse agonists, according to cAMP generation, at the β 2-adrenergic receptor ICI118551 and propranolol, were demonstrated to recruit β -arrestin2 and activate MAP kinase through this pathway (Azzi et al., 2003). Furthermore, amphetamine-induced hyperactivity, a traditionally used pre-clinical psychosis model, has been shown to be dependent on the arrestin pathway possibly by scaffolding D2-like receptors with the kinase Akt leading to the dephosphorylation and subsequent reduction in Akt activity (Beaulieu et al., 2004, 2005, 2007). Importantly, the hyperactivity appears independent of the cAMP cascade. This indicates that alternative pathways involving β -arrestins rather than G-protein induced changes in cAMP levels might contribute to the anti-psychotic properties of D2 antagonism.

In this study we develop and characterize a BRET based assay reporting β -arrestin2 recruitment to the D2 receptor. Using this assay we have profiled a selection of well-described drugs used in the treatment of Parkinson's disease and schizophrenia in order to evaluate if the efficacy and potency of these compounds to recruit β -arrestin2 differs from the efficacy and potency described in the literature using traditional GTP γ S and cAMP assays.

2. Materials and methods

2.1. Cell culturing

Human embryonic kidney (HEK) 293 cell lines (American Type Culture Collection, Manassas, VA, USA) were maintained in Dulbecco's modified eagle medium (DMEM; Invitrogen, Taastrup, Denmark) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 1% penicillin–streptomycin (penicillin G sodium: 10 U/mL, streptomycin

sulfate 10 mg/mL in 0.85% saline; Invitrogen) and 2 mM L-glutamine (Invitrogen). Cells were cultured at 37 °C, in 90% humidity and 5% CO₂.

2.2. Mammalian expression plasmids

The open reading frame (ORF) for the short transcriptional variant of human dopamine receptor D2 (hD2_S) was fused N-terminally to a previously described signal flag sequence (Guan et al., 1992) and C-terminally to an optimized version of Renilla Luciferase, Rluc8 (Loening et al., 2006). The amino acid linker sequence separating hD2_S and Rluc8 was ATGLRSRAQASNSAVDGTAGPVAT. The fusion construct D2_S-Rluc8 was cloned into the mammalian expression vector pcDNA 3.1(+) (Invitrogen). Total vector size was 7.7 kb. The analogue construct with the long transcriptional variant of the human dopamine receptor D2 (hD2_L) was constructed similarly and named D2_L-Rluc8. The fusion construct β-arrestin2-mVenus was cloned into pHsCXW (Leander et al., 2005) using standard PCR cloning techniques. Total vector size was 9.4 kb. The ORF for human β-arrestin2 (GenBank accession number NM_004313) was PCR amplified and fused in a second PCR reaction via a coding sequence for the amino acid sequence GAGALAT to the N-terminus of the Green Fluorescent Protein variant monomeric Venus (mVenus) (Nagai et al., 2002). The β-arrestin2 double mutant, β-arrestin2(R393E,R395E)-mVenus was generated by site directed mutagenesis on β-arrestin2-mVenus. All vector inserts were verified by sequencing (MWG DNA, Ebersberg, Germany).

2.3. Transfections

Transfections with DNA were done with Lipofectamine 2000 (Invitrogen), according to the manufacturers protocol. The ratio of Lipofectamine 2000 to DNA ratio was 4 (μL to μg). Where nothing else is stated 500 ng of DNA was used per 10 cm² of cells grown to 90–100% confluency at the time of transfection. For BRET and immunofluorescence experiments transfected cells were split 6–18 h post-transfection and re-seeded at approximately 40% and 10% confluency, respectively.

2.4. Chemical compounds and vehicle

All chemical compounds were synthesized by H. Lundbeck A/S, Denmark and dissolved in DMSO to a stock concentration of 10 mM. For BRET measurements compounds were diluted to the appropriate concentration in buffer composed of PBS with Ca²⁺ and Mg²⁺ (Invitrogen) supplemented with 0.1% w/v sucrose (Sigma-Aldrich, Broendby, Denmark) and 0.2 μM ascorbic acid (Sigma-Aldrich). Control buffer was identical except that 2% v/v DMSO (Sigma-Aldrich) was added corresponding to the DMSO carry-over in test solutions with 10 μM compound.

2.5. BRET measurements and calculations

Forty-eight hours post-transfection cells were harvested by trypsinization (1 mL per 15 cm culture dish for ~45 s) (Invitrogen). All subsequent steps were performed at room temperature. Cells were re-suspended in culture medium and centrifuged at 900g for 3 min and subsequently re-suspended in PBS with Ca²⁺ and Mg²⁺ containing 0.01% sucrose (w/v) to a density of approximately 100,000–200,000 cells/mL. Cells were left in suspension for 45–75 min before incubating with compounds for 16 min (if nothing else is stated). Subsequently, cells were mixed with coelenterazine *h* (Invitrogen) to a final concentration of 2 μM and luminescence was measured immediately using a VICTOR Light Luminescence Counter (Perkin-Elmer, Waltham, MA, USA). Luminescence from each well was measured twice; first through a 522.5–547.5 nm bandpass filter and then through a 445–485 nm bandpass filter (Perkin-Elmer). Within each experiment measurement of each condition was done in duplicate or triplicate. The BRET ratio was calculated as the long wavelength

emission divided by short wavelength emission and expressed as the relative change compared to vehicle treated cells (Pfleger et al., 2006).

2.6. Immunofluorescence and confocal microscopy

HEK293 cells transiently transfected using Lipofectamine 2000 reagent were seeded the day after transfection on coverslips coated with poly-L-ornithine (Sigma-Aldrich) in PBS buffer. One to two days after seeding, the cells were fixed in 4% formaldehyde solution in PBS buffer, blocked in blocking solution (5% goat serum in PBS buffer), incubated with primary antibody (M1 anti-FLAG 1:1000; Sigma) and secondary antibody (goat anti-mouse Alexa Fluor 488; Invitrogen). For permeabilization cells were treated with 0.3% saponin for 20 min at room temperature. Stained cells were visualized using a Zeiss (Oberkochen, Germany) LSM510 confocal laser-scanning microscope with an oil immersion 63 $\times$ objective. The Alexa Fluor 488 and mVenus was excited with the 488 nm laser line from an argon-krypton laser and the emitted light was detected using a 505–550 nm bandpass filter.

2.7. Data analysis and statistical methods

All statistical analysis, regressions and curve fitting were performed using GraphPad Prism version 4 (GraphPad Software, San Diego, CA, USA). A normal distribution of sample data was assumed. Student's *t*-test was used to compare average of two groups and one-way ANOVA was used to compare averages of multiple groups. The null hypothesis was rejected at the $p < 0.05$ level. All BRET experiments were performed at least three times and results are presented as an average $\pm$ standard error of mean (s.e.m). Confocal pictures are representative. For dose–response experiments, individual curve fits were obtained by using the sigmoidal dose–response variable slope algorithm. For compound efficacy calculations the obtained efficacy for each individual experiment was normalized to dopamine and expressed as the mean $\pm$ s.e.m. Similarly, individual log(EC₅₀) and log(IC₅₀) values were obtained and expressed as the mean $\pm$ s.e.m. K_b values were obtained by using a generalized Cheng–Prusoff equation (Leff and Dougall, 1993).

3. Results

3.1. Dopamine induced recruitment of human β -arrestin2 to the human dopamine receptor D2 measured by BRET

To quantify ligand induced coupling of the D2 receptor to β -arrestin2, a Bioluminescence Resonance Energy Transfer (BRET) system was utilized allowing us to characterize the ability of different compounds to recruit β -arrestin2 to the short splice variant of the human D2 receptor (hD2_S) as well as the long transcriptional variant hD2_L. Importantly, previous work has demonstrated the usefulness of BRET as a pharmacological tool to characterize β -arrestin recruitment to both class A and class B GPCRs. We used β -arrestin2 over β -arrestin1 because the D2 receptor belongs to the group of class A GPCRs lacking the C-terminal clusters of threonines and serines, and thus exhibits a higher affinity for β -arrestin2 than for β -arrestin1 (Oakley et al., 2000; Kim et al., 2001). In BRET experiments, it is possible to make relative quantifications of molecular interactions if the donor and acceptor are within a spatial range of 0–10 nm apart. For class A receptors, however, the detection window is smaller, which is most likely due to the transient nature of β -arrestin interaction with the receptor (Vrecl et al., 2004; Hamdan et al., 2005).

To study the recruitment of β -arrestin2 to hD2_S upon ligand binding, HEK293 cells were transiently transfected with luciferase-tagged hD2_S (D2_S-Rluc8) and mVenus-tagged β -arrestin2 (β -arrestin2-mVenus). In HEK293 cells, β -arrestin2-mVenus showed a uniform cytoplasmic localization (Fig. 1A). In contrast, immunocytochemical staining of N-

terminally flag-tagged D2_S-Rluc8 revealed a large fraction of D2_S-Rluc8 localized to the plasma membrane (Fig. 1A).

Previous work has demonstrated that fluorescence microscopy can be used to assess the translocation of GFP tagged β -arrestin2 to D2-like receptors (Kim et al., 2001). This approach suffers a drawback: quantification of translocation events is difficult and time consuming. Therefore, a method for relative quantification of β -arrestin2 recruitment to the hD2_S receptor was established. In our experimental setup, BRET was measured upon addition of the Renilla luciferase substrate coelenterazine *h* to HEK293 cells transiently expressing the BRET donor D2_S-Rluc8 and the BRET acceptor β -arrestin2-mVenus. This approach has proven successful in quantifying β -arrestin2 recruitment to other GPCRs, in particular in studies with class B receptors that exhibit prolonged interaction times with β -arrestin2 (Hamdan et al., 2005). The BRET detection method utilized in the present study exhibits a spectral overlap between the emission spectrum from the catalysis of coelenterazine *h* and the emission wavelengths measured from mVenus. Consequently, excessive presence of D2_S-Rluc8 will result in luciferase molecules not engaging in BRET and thereby attenuating the BRET signal. We therefore first tested the effect of varying the DNA mass ratio of the BRET donor (D2_S-Rluc8) and BRET acceptor (β -arrestin2-mVenus). As shown in Fig. 1B, increasing mass ratios of β -arrestin2-mVenus to D2_S-Rluc8 clearly increased the BRET ratio in response to 10 μ M dopamine. Moreover, the increase was saturable in agreement with a specific interaction (Fig. 1B). In our subsequent experiments, we chose to express the constructs at a mass ratio of 10:1 (β -arrestin2-mVenus:D2_S-Rluc8). The absolute level of exogenous expressed D2_S-Rluc8 and β -arrestin2-mVenus appeared not to influence the increase in the BRET ratio induced by 10 μ M dopamine; hence, cells transfected with varying amounts of vector DNA in a constant mass ratio of 10:1 (β -arrestin2-mVenus:D2_S-Rluc8) did not affect the dopamine induced increase in BRET ratio (Fig. 1C). With a similar approach we also observed the EC₅₀ value of dopamine to remain constant with varying amounts of vector DNA used for transfection (Fig. 1D), indicating that spare receptors are not present in the system. Furthermore, the agonist induced recruitment of β -arrestin2-mVenus to D2_S-Rluc8 was fast and sustained. After addition of 10 μ M dopamine, maximal BRET ratios could be measured after approximately 5 min (Fig. 1E) without decline within 20 min. We also observed that vehicle treated cells exhibited a slow increase in BRET ratio over time (Fig. 1E). Nevertheless, because the compound and vehicle induced changes in BRET ratio were measured at the same time point within each experiment this slow rise in basal BRET levels did not affect the assessment of compound induced changes in the BRET ratio.

3.2. Agonist induced recruitment of β -arrestin2 to hD2_S characterized by BRET measurements

After having established a method for measuring ligand induced recruitment of β -arrestin2 to hD2_S in HEK293 cells, we investigated concentration-response relationships for well-characterized agonists at the D2 receptor (Fig. 2 and Table 1) (Newman-Tancredi et al., 2002; Gay et al., 2004; Urban et al., 2007b). Dopamine and the D2-class selective full agonist quinpirole both acted as full agonists with EC₅₀ values of 49 nM (95% C.I.: 43–57 nM) and 75 nM (95% C.I.: 58–98 nM), respectively (Fig. 2A and Table 1). The anti-parkinsonian drug pramipexole, which acts at D2-like receptors, also behaved as a full agonist (Fig. 2A and Table 1), whereas three other anti-parkinsonian drugs, pergolide, ropinirole and bromocriptine all behaved as partial agonists with efficacies of 90±1%, 74±2% and 50±4% relative to dopamine, respectively (Fig. 2A and C and Table 1). The D1/D2 dopamine receptor non-selective partial agonist, apomorphine was a partial agonist with an efficacy of 84±2% relative to dopamine (Fig. 2C and Table 1). The aporphine stereoisomers RNPA and SNPA acted as partial agonists with an efficacies 92±7% and 14

$\pm 2\%$ relative to dopamine (Fig. 2E and Table 1). The D2-like receptor selective partial agonists terguride and S(–)-3-(3-hydroxyphenyl)-N-propylpiperidine (3PPP) also acted as partial agonists with efficacies of 20 ± 2 and $24 \pm 6\%$ relative to dopamine, respectively (Fig. 2C and E and Table 1). We also tested the D1/D2 receptor agonist dihydrexidine. Unexpectedly, this compound inhibited luciferase activity in a D2 receptor independent manner (data not shown) and consequently, we were unable to determine the pharmacological properties of this compound in our assay. In contrast to these agonists, the anti-psychotic drug and D2 receptor inverse agonist clozapine (Wilson et al., 2001) did not recruit β -arrestin2 to hD2_S in a concentration dependent manner (Fig. 2A). The potencies and hill coefficients of the listed compounds are given in Table 1. The ranking order of potencies for the compounds tested in the assay was: RNPA > aripiprazole > terguride > pergolide > apomorphine > bromocriptine > SNPA > pramipexole > 3PPP > dopamine > quinpirole > ropinirole.

To increase the sensitivity of the BRET assay and to confirm that clozapine was unable to recruit β -arrestin2 to hD2_S, we took advantage of a previously described double mutant in β -arrestin2(R393E,R395E) that has been reported to exhibit prolonged interaction with both class A and class B GPCRs (Vrecl et al., 2004; Jorgensen et al., 2007). Using β -arrestin2(R393E,R395E)-mVenus in our BRET experiments, we observed an approximately twofold increase in the BRET ratio induced by the full agonists. The partial agonist terguride exhibited a higher efficacy ($p < 0.01$) as did the partial agonist bromocriptine ($p < 0.05$) compared to the assay with wild-type β -arrestin2-mVenus (Table 1). The rest of the tested compounds did not show any statistically significant difference when comparing their efficacy with the efficacies obtained in the wild-type assay. Dopamine, pramipexole and bromocriptine were observed to have EC₅₀ values that were significantly different from the EC₅₀ value observed in the wild-type assay ($p < 0.01$, $p < 0.05$ and $p < 0.01$, respectively) (Table 1). The change in potency for dopamine and pramipexole was only 0.7-fold whereas bromocriptine exhibited a 5.4-fold change in potency. The rest of the tested compounds did not show any statistically significant difference when comparing their potency with the potencies obtained in the wild-type assay. As in the wild-type assay, clozapine was not able to recruit the double mutant form of β -arrestin2 to hD2_S in a concentration dependent manner (Fig. 2B).

3.3. Inhibition of dopamine dependent β -arrestin2 recruitment to the human dopamine D2 receptor by commonly used anti-psychotics

To investigate the possibility that anti-psychotics act differentially on dopamine induced β -arrestin2 recruitment to hD2_S, we investigated a selection of anti-psychotics for their ability to inhibit this recruitment. We determined the EC₈₅ concentration of dopamine to be approximately 280 nM in the assay and we decided to use this concentration of dopamine in the experiment. Accordingly, we incubated HEK293 cells transiently transfected with the BRET vector constructs for 5 min with different antipsychotics in increasing concentrations followed by exposure to 280 nM dopamine for an additional 15 min. Haloperidol, sertindole, olanzapine, clozapine and ziprasidone were all capable of fully inhibiting β -arrestin2 recruitment to hD2_S whereas aripiprazole showed a tendency towards less than full inhibition of β -arrestin2 recruitment (Fig. 3A and Table 2). This tendency was even more pronounced when performing the experiment with β -arrestin2(R393E,R395E)-mVenus (Fig. 3B and Table 2). These observations indicate that aripiprazole exhibits, albeit limited, partial agonistic properties in recruiting β -arrestin2 to hD2_S. We confirmed this observation by testing if aripiprazole was able to induce recruitment of β -arrestin2 to hD2_S, and found the compound to be a partial agonist with an efficacy of $17 \pm 5\%$ compared to dopamine (Fig. 2E and Table 1). The apparent log IC₅₀ values, the estimated K_b values and hill coefficients

for aripiprazole, haloperidol, sertindole, olanzapine, clozapine and ziprasidone have been listed in Table 2.

3.4. Correlation between ligand induced recruitment of β -arrestin2 to the short and the long transcriptional variant of the human dopamine D2 receptor

To investigate if the pharmacological data on β -arrestin2 recruitment to hD2_S can be extended to hD2_L we set up an analogue BRET assay using D2_L-Rluc8 with β -arrestin2-mVenus. Using this assay we determined the potencies of the full agonist as well as the partial agonists that were found to have efficacies >20% compared to dopamine in their ability to recruit β -arrestin2 to hD2_S. We found a strong direct correlation between the potency of a given compound in recruiting β -arrestin2 to hD2_S and to hD2_L (Fig. 4A). We also determined the efficacy of these compounds in the assay with D2_L-Rluc8 and found these to closely match the efficacy determined in the assay with D2_S-Rluc8 (Fig. 4B). It is noteworthy that some compounds exhibited discrepancies to the general correlations. Bromocriptine had an approximately sixfold lower potency for β -arrestin2 recruitment to hD2_L compared to hD2_S and pramipexole appeared to be a partial agonist in recruiting β -arrestin2 to hD2_L.

3.5. Evaluation of typical and atypical anti-psychotics

The anti-psychotic drug clozapine did not recruit β -arrestin2 to hD2_S; however, this does not exclude that this could be the case for other anti-psychotics. Accordingly, we tested a larger selection of anti-psychotic compounds for their putative intrinsic efficacy towards β -arrestin2 recruitment to hD2_S as well as to hD2_L in the BRET assay. As shown in Fig. 4C, only small deviations from baseline were observed and only one compound, molindone, induced small significant changes in the BRET signal with both hD2_S and hD2_L (Student's *t*-test, *p* < 0.05).

4. Discussion

We have established a cellular assay for determination of β -arrestin2 recruitment to the hD2_S and hD2_L receptors. Furthermore, we have validated the use of β -arrestin2(R393E,R395E) for pharmacological characterization of β -arrestin2 recruitment to the hD2_S receptor. This mutant improves the detection window approximately twofold in agreement with previous observations for other GPCRs (Vrecl et al., 2004), and with only small or no changes in compound potency compared to the wild-type assay. The exception to this was bromocriptine, which had a 5.4-fold lower potency and a slightly higher efficacy in the assay that employed β -arrestin2(R393E,R395E). The reason for this is presently unknown but we speculate that the prolonged interaction of β -arrestin2(R393E,R395E) with the D2 receptor may modulate the pharmacological properties of bromocriptine at the receptor.

Using hD2_L in the BRET assay we found that there was a strong agreement between potencies and efficacies determined for β -arrestin2 recruitment to hD2_S and hD2_L. The exceptions to this were bromocriptine, which was observed to exhibit a ~sixfold lower potency towards recruitment of β -arrestin2 to hD2_L compared to hD2_S and pramipexole which were determined to be a partial agonist with a high efficacy at hD2_L as opposed to being a full agonist at hD2_S. The reason for this is presently unknown. We observed the agonist induced BRET detection window to be slightly smaller when using hD2_L compared to hD2_S. This phenomenon could explain the small differences in ligand efficacy that was observed, and why pramipexole acted as a partial agonist at hD2_L.

We employed the assay to test a selection of well-characterized D2-like receptor agonists and partial agonists for their ability to recruit β -arrestin2 to hD2_S in a concentration dependent manner. Consistent with previous findings on G-protein coupling to hD2_S quinpirole and pramipexole acted as full agonists in their ability to recruit β -arrestin2 (Newman-Tancredi et al., 2002). In contrast, pergolide and ropinirole acted as partial agonists with a high efficacy, as opposed to their ascribed role as full agonist (Newman-Tancredi et al., 2002). Based on the presumably low expression level of D2_S-Rluc8 we speculate that this discrepancy is due to a lack of spare receptors in the β -arrestin2 recruitment assay described here. Apomorphine, bromocriptine and terguride were all partial agonists with an efficacy order of apomorphine > bromocriptine > terguride, also in agreement with the literature on G-protein coupling (Newman-Tancredi et al., 2002). The relative ranking of the potencies of these compounds was found to be terguride > pergolide $\approx$ apomorphine > bromocriptine > pramipexole $\approx$ dopamine > quinpirole $\approx$ ropinirole. With the exception of bromocriptine, which was less potent than pergolide and apomorphine in recruiting β -arrestin2 to hD2_S the rank order also agrees with the literature on G-protein coupling to hD2_S within a narrow twofold range difference in EC₅₀ values (Newman-Tancredi et al., 2002). Aripiprazole and 3PPP have previously been found to be highly potent partial agonists with low efficacy at D2 mediated inhibition of cAMP accumulation (Burris et al., 2002) and both acted as highly potent partial agonists with low efficacy in the β -arrestin2 recruitment assay. RNPA and SNPA have been described as being full agonists with respect to hD2_L mediated inhibition of cAMP, with RNPA being the most potent of the two. In addition, RNPA and SNPA have been observed to exhibit functional selective properties with regard to other pathways downstream of the hD2_L receptor (e.g. MAP kinase and GIRK channel activation) (Gay et al., 2004). However, in an other study SNPA was determined to be a partial agonist with regard to cAMP signaling (Kilts et al., 2002). This discrepancy could arise from spare receptor effects in the heterologous expression system where SNPA was observed to be a full agonist. In the present study, RNPA and SNPA acted as partial agonists with a high and low efficacy, respectively, towards hD2_S mediated β -arrestin2 recruitment and with RNPA being the more potent of the two. In summary, we find no strong indications that the tested agonists and partial agonists are able to stimulate recruitment of β -arrestin2 to hD2_S in a functional selective manner compared to G-protein coupling and inhibition of cAMP production.

With the exception of the partial agonist aripiprazole, all other anti-psychotics (clozapine, haloperidol, olanzapine, sertindole and ziprasidone) tested in our assay fully prevented dopamine induced recruitment of β -arrestin2 to hD2_S. These experiments indicate that in HEK293 cells these anti-psychotics do not exhibit functional selective properties towards β -arrestin2 recruitment. Furthermore, we tested a broader selection of anti-psychotics at 10 μ M concentrations in our BRET assay with hD2_S/hD2_L and β -arrestin(R393E,R395E). Only molindone mediated a significant increase in the BRET signal with both the hD2_S and hD2_L receptors although the increase in the BRET signal was limited. Taken together these observations are perhaps somewhat surprising given the studies of a number of other receptors for which biased agonism towards β -arrestin2 recruitment has been observed (Azzi et al., 2003; Violin and Lefkowitz, 2007; Wisler et al., 2007; Jorgensen et al., 2007). We cannot rule out that the anti-psychotics stabilize a conformation of the hD2_S and hD2_L receptors that indeed is able to recruit β -arrestin2 but somehow inadequate for BRET to occur. This possibility is unlikely, however, because the long flexible linker used to separate hD2_S/hD2_L and Rluc8 would be expected to allow for detection of BRET independent on the actual receptor conformation. To support this idea we also established an assay in which the BRET donor (Rluc8) was fused to β -arrestin2 and the acceptor (mVenus) to hD2_S (data not shown). This assay, with its relative higher level of hD2_S receptor expression, also did not reveal any capacity of the tested anti-psychotics to induce recruitment of β -arrestin2 to hD2_S.

Based on our observations we hypothesize that β -arrestin2 and G-protein coupling at the D2 receptor are tightly associated and orchestrated events. Parallel effects on cAMP accumulation and on the putative β -arrestin/Akt signaling pathway suggested by Beaulieu et al. (2007) makes it very difficult at present to discern which of these pathways are responsible for the positive effects as well as the side-effects of anti-psychotics mediated through D2-like receptors. Using our novel BRET assay it may be possible to identify compounds that could be used to further unravel the role of D2 receptors and its different downstream signaling pathways in schizophrenia and Parkinson's disease.

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Fig. 1.

The vectors encoding the BRET donor D2S-Rluc8 and the BRET acceptor β -arrestin2-mVenus were transiently expressed in HEK293 cells. (A) Confocal microscopy of cells expressing (from left to right) β -arrestin2-mVenus and D2S-Rluc8 tagged with an N-terminal flag epitope and visualized with M1 antibody with and without permeabilization. (B) The amount of vectors encoding the BRET acceptor, β -arrestin2-mVenus, to the BRET donor, D2S-Rluc8, was varied and cells were stimulated with 10 μ M dopamine for 16 min before BRET was measured. There was no statistical significant difference in the BRET ratio between expressing the BRET acceptor 10-fold and 15-fold relative to the BRET donor (Student's *t*-test). Data fitted to one-phase exponential association. (C) The amount of vector

encoding β -arrestin2-mVenus relative to the amount of vector encoding D2_S-Rluc8 was held constant (10 to 1) while the total amount of DNA (per 10 cm² cells) used for transfection was varied. There was no statistical significant difference between the averages of 10 μ M dopamine induced increase in BRET (one-way ANOVA). Ligand induced changes in the BRET signal expressed as the increase relative to vehicle treated cells (set equal to 1) ($n = 3$) for each condition, mean $\pm$ s.e.m. (D) The amount of vector encoding β -arrestin2-mVenus relative to the amount of vector encoding D2_S-Rluc8 was held constant (10 to 1) while the total amount of DNA (per 10 cm² cells) used for transfection was varied. On the basis of individual dopamine concentration response curves the dopamine log EC₅₀ values were determined. There was no statistical significant difference between the averages of dopamine log EC₅₀ values (one-way ANOVA) ($n = 3$) for each condition, mean $\pm$ s.e.m (E). The amount of vector encoding β -arrestin2-mVenus relative to the amount of vector encoding D2_S-Rluc8 was held constant (10 to 1) and the BRET ratio was measured in milliBRET (mBRET) over time in cells treated with 10 μ M dopamine (●) or vehicle (○).

Fig. 2.

(A, C and E) Agonist concentration–response curves of HEK293 transiently transfected with D2_S–Rluc8 and β -arrestin2–mVenus (10-fold surplus) treated with indicated ligands. (B, D and F) Agonist concentration–response curves of HEK293 transiently transfected with D2_S–Rluc8 and β -arrestin2(R393E,R395E)–mVenus (10-fold surplus) treated with indicated ligands. (G) The log EC₅₀ values obtained for 3PPP, apomorphine, aripiprazole, bromocriptine, dopamine, pergolide, pramipexole, quinpirole, RNPA, ropinirole, SNPA, terguride with D2_S–Rluc8 and WT β -arrestin2, respectively, β -arrestin2(R393E,R395E)–mVenus were correlated to a linear function ($p < 0.0001$; $r^2 = 0.82$, slope = 0.97; slope 95%

C.I.: 0.65–1.28). Ligand induced changes in the BRET signal expressed relative to the maximal response to dopamine ($n = 4$ –11) for each condition, mean $\pm$ s.e.m.

Fig. 3.

(A) Antagonist-mode concentration–response curves of HEK293 transiently transfected with D2_S–Rluc8 and β -arrestin2–mVenus (10-fold surplus) treated with indicated ligands. (B) Antagonist-mode concentration–response curves of HEK293 transiently transfected with D2_S–Rluc8 and β -arrestin2(R393E,R395E)–mVenus (10-fold surplus) treated with indicated ligands. Ligand induced changes in the BRET signal expressed relative to the response to 280 nM dopamine ($n=4-5$) for each condition, mean $\pm$ s.e.m.

Fig. 4.

(A) The log EC_{50} values for β -arrestin2 recruitment to $hD2_L$ were determined for ligands with efficacies of >20% (3PPP, apomorphine, bromocriptine, dopamine, pergolide, pramipexole, quinpirole, RNPA, ropinirole, terguride) and correlated to the log EC_{50} values for β -arrestin2 recruitment to $hD2_S$ ($p < 0.0001$; $r^2 = 0.92$, slope = 1.13; slope 95% C.I.: 0.85–1.40). (B) The efficacy of the indicated ligands for recruitment of β -arrestin2 to $hD2_S$ and $hD2_L$ was compared. Small significant changes in the efficacy of terguride, ropinirole and pramipexole were observed (* $p < 0.05$, Student's t -test). (C) BRET measured in HEK293 cells transiently transfected with $D2_S$ -Rluc8 and β -arrestin2(R393E,R395E)-mVenus (7.5- to 10-fold surplus) or $D2_L$ -Rluc8 and β -arrestin2(R393E,R395E)-mVenus (7.5- to 10-fold surplus) and treated with 10 μ M of the indicated ligands. * Indicates that the ligand induced a change in the BRET ratio significant different from baseline with both $hD2_S$ and $hD2_L$ ($p < 0.05$, Student's t -test relative to baseline for each receptor). Ligand induced changes in the BRET signal expressed relative to the response to 10 μ M dopamine ($n = 3$) for each condition, mean $\pm$ s.e.m.

Table 1
Pharmacological key figures of ligand induced recruitment of β -arrestin2 and β -arrestin2(R393E,R395E) to the hD_{2S} receptor

	log EC ₅₀			Efficacy (% of dopamine)			Hill-slope				
	β-Arrestin2 WT	β-Arrestin2 (R393E,R395E)	p-Value	Fold change	β-Arrestin2 WT	β-Arrestin2 (R393E,R395E)	p-Value	Fold change	β-Arrestin2 WT	β-Arrestin2 (R393E,R395E)	p-Value
3PPP	-7.46 ± 0.41	-6.86 ± 0.19	ns		23.56 ± 6.44	22.59 ± 1.66	ns		1.05 ± 0.23	0.71 ± 0.09	ns
Apomorphine	-8.29 ± 0.04	-8.34 ± 0.04	ns		83.68 ± 2.17	79.79 ± 2.57	ns		1.04 ± 0.11	1.11 ± 0.06	ns
Aripiprazole	-8.99 ± 0.18	-8.20 ± 0.31	ns		17.08 ± 5.07	11.22 ± 1.69	ns		0.68 ± 0.39	0.77 ± 0.14	ns
Bromocriptine	-7.79 ± 0.11	-7.06 ± 0.15	<0.01	5.4	49.70 ± 3.95	60.11 ± 1.92	<0.05	1.2	1.41 ± 0.16	1.33 ± 0.15	ns
Dopamine	-7.31 ± 0.03	-7.46 ± 0.03	<0.01	0.7	100.00	100.00	–		0.87 ± 0.07	0.90 ± 0.06	ns
Pergolide	-8.52 ± 0.04	-8.52 ± 0.04	ns		89.97 ± 0.95	95.53 ± 4.51	ns		1.12 ± 0.14	1.02 ± 0.07	ns
Pramipexole	-7.55 ± 0.05	-7.69 ± 0.04	<0.05	0.7	104.56 ± 3.45	102.24 ± 4.75	ns		0.72 ± 0.12	0.86 ± 0.03	ns
Quinpirole	-7.12 ± 0.05	-7.26 ± 0.06	ns		102.69 ± 3.37	104.93 ± 3.88	ns		0.85 ± 0.03	0.84 ± 0.03	ns
RNPA	-9.54 ± 0.07	-9.83 ± 0.26	ns		92.39 ± 7.01	91.53 ± 3.30	ns		0.93 ± 0.06	0.86 ± 0.13	ns
Ropinrole	-6.98 ± 0.10	-7.04 ± 0.06	ns		74.43 ± 1.81	82.58 ± 2.73	ns		1.46 ± 0.50	0.88 ± 0.03	ns
SNPA	-7.64 ± 0.13	-7.41 ± 0.14	ns		14.09 ± 2.42	13.45 ± 1.07	ns		1.59 ± 0.33	1.42 ± 0.30	ns
Terguride	-8.90 ± 0.31	-8.73 ± 0.05	ns		20.17 ± 1.62	29.63 ± 2.02	<0.01	1.5	1.52 ± 0.36	1.58 ± 0.20	ns

Agonist efficacy was calculated by normalizing to E_{max} for dopamine within each experiment and expressed as mean ± s.e.m. The log EC₅₀ and hill coefficients were calculated for each individual concentration–response experiments and expressed as mean ± s.e.m. ($n = 4-11$). Average efficacy, log EC₅₀, and hill-slope values obtained with β -arrestin2 were compared with the corresponding values obtained with β -arrestin2(R393E,R395E) by Student's *t*-test.

Table 2

Pharmacological key figures of ligand induced inhibition of recruitment of β -arrestin2(R393E,R395E) to the hD2_S receptor

	Apparent log IC ₅₀			Estimated K _b (general Cheng–Prusoff)			Hill-slope		
	β-Arrestin2 WT (R393E,R395E)	p-Value	Fold change	β-Arrestin2 WT (R393E,R395E)	p-Value	Fold change	β-Arrestin2 WT (R393E,R395E)	p-Value	
Aripiprazole	-7.87 ± 0.11	ns		-8.75 ± 0.11	ns		-1.65 ± 0.10	0.0389	
Clozapine	-6.26 ± 0.04	0.0263	1.3	-7.15 ± 0.04	0.0263	1.3	-1.11 ± 0.07	0.047	
Haloperidol	-8.61 ± 0.11	ns		-9.50 ± 0.11	ns		-1.14 ± 0.06	ns	
Olanzapine	-7.37 ± 0.07	ns		-8.26 ± 0.07	ns		-1.40 ± 0.26	ns	
Sertindole	-7.32 ± 0.08	ns		-8.20 ± 0.08	ns		-1.44 ± 0.17	ns	
Ziprasidone	-8.05 ± 0.08	ns		-8.93 ± 0.08	ns		-1.67 ± 0.17	ns	

The apparent log IC₅₀ and hill-slope were calculated for each individual concentration–response experiment and expressed as mean ± s.e.m. ($n = 4-5$). K_b values were subsequently estimated using a general Cheng–Prusoff equation and expressed as mean ± s.e.m.