Pharmacological analysis of a dopamine D_{2Short}:$G_{\alpha o}$ fusion protein expressed in Sf9 cells

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Abstract **A dopamine D_{2Short} receptor:$G_{\alpha o}$ fusion protein was expressed in Sf9 cells using the baculovirus expression system. [^{3}H]Spiperone bound to D_{2Short}:$G_{\alpha o}$ with a $pK_d \approx 10$. Dopamine stimulated the binding of [^{35}S]guanosine-5′-*O*-(3-thio)triphosphate (GTPγS) to D_{2Short}:$G_{\alpha o}$ expressed with $G\beta_1\gamma_2$ ($E_{max} > 460\%$; pEC_{50} 5.43 ± 0.06). Most of the putative D_2 antagonists behaved as inverse agonists (suppressing basal [^{35}S]GTPγS binding) at D_{2Short}:$G_{\alpha o}$/$G\beta_1\gamma_2$ although (−)-sulpiride and ziprasidone were neutral antagonists. Competition of [^{3}H]spiperone binding by dopamine and 10,11-dihydroxy-*N*-*n*-propylnorapomorphine revealed two binding sites of different affinities, even in the presence of GTP (100 μM). The D_{2Short}:$G_{\alpha o}$ fusion protein is therefore a good model for characterising D_2 receptors.**

Key words: D_2 dopamine receptor; G protein-coupled receptor; Fusion protein; Baculovirus; [^{3}H]Spiperone binding; [^{35}S]Guanosine-5′-*O*-(3-thio)triphosphate binding

1. Introduction

The mechanisms by which ligands interact with and activate G protein-coupled receptors (GPCR) remain poorly understood. This is partly due to the fact that several parameters can affect the response observed upon GPCR stimulation by an agonist, including the number of receptors (R), the number of G proteins (G) and the R/G ratio [1,2]. Further complexity arises from the finding that a single GPCR can activate distinct G proteins within the same cell system [3,4]. Fusion proteins, which covalently link the receptor to the G_α subunit of the G protein, have been introduced as an alternative approach which might help in understanding the mechanisms of interaction and activation of receptors and G proteins by different ligands, without the potential confounding effect of differences in R/G ratio. Hence fusion proteins between diverse GPCRs and their cognate G proteins have been used to study receptor/G protein interaction [5–7].

The D_2 dopamine receptor, a member of the dopamine receptor family (which comprises five receptors, D_1–D_5), is a prototypical GPCR, which interacts with and signals via G proteins of the $G_{i/o}$ family. Alternative splicing of the D_2 receptor mRNA results in two isoforms of the receptor, termed D_{2S} (short) and D_{2L} (long) [8]. The D_{2S} receptor was shown to couple preferentially to G_{i1} over G_{i2} when expressed in Sf9 cells [9] and distinct roles for G protein subunits in the modulation of cAMP accumulation and calcium mobilisation by D_2 receptors were described [10,11]. It was also suggested that the 29 amino acid insert of D_{2L} might confer selectivity for interaction with G_{i2} [12]. Recently, we reported the regulation of G protein selectivity by agonists for the rat and for the human D_{2L} receptor using the baculovirus expression system [13,14]. These observations suggest differences in the molecular mechanisms of the interaction of the D_2 receptors with different G proteins, which may account for different signalling and therefore different functions. Further insights into mechanisms of action of agonists at GPCRs may be obtained using the receptor–G protein fusion strategy. However, no report has yet described a functional fusion protein between the D_2 dopamine receptor and a G protein.

The aim of this study was to analyse the pharmacological profile of a fusion protein involving the human D_{2S} receptor and a rat $G_{\alpha o}$ subunit of the G protein, using the baculovirus expression system. Our results show that such a construct presents characteristics similar to those of the D_2 receptor expressed in other heterologous systems. In addition, our data also suggest that the D_{2S} receptor engaged in a fusion with the $G_{\alpha o}$ subunit can exhibit constitutive activity, as illustrated by the inverse agonist activity of some putative D_2 receptor antagonists.

2. Materials and methods

2.1. Materials

Bromocriptine, (+)-butaclamol, clozapine, dopamine, haloperidol, (−)-quinpirole, *m*-tyramine, *p*-tyramine, *S*-(−)-3-(3-hydroxyphenyl)-*N*-propylpiperidine (3-PPP), *R*-(+)-3-PPP, *R*-(−)-10,11-dihydroxy-*N*-*n*-propylnorapomorphine (NPA) were purchased from Sigma (St. Louis, MO, USA). Aripiprazole and ziprasidone were generous gifts from Psychiatry CEDD, GlaxoSmithKline (Harlow, UK). (+)-AJ76 and (+)-UH232 were from Tocris (Bristol, UK). [^{3}H]Spiperone (15–30 Ci/mmol) and [^{35}S]guanosine-5′-*O*-(3-thio)triphosphate (GTPγS, 1099–

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Abbreviations: D_2, dopamine D_2 receptor; $G_{i/o}$, heterotrimeric GTP binding protein type i or o; GPCR, G protein-coupled receptor; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; NPA, 10,11-dihydroxy-*N*-*n*-propylnorapomorphine; PCR, polymerase chain reaction; PTX, *Bordetella pertussis* toxin; 3-PPP, 3-(3-hydroxyphenyl)-*N*-propylpiperidine

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1148 Ci/mmol) were from Amersham International (Buckinghamshire, UK). All other reagents were obtained as indicated.

2.2. Construction of D_{2S}:$G_{\alpha o}$ fusion protein

The D_{2S}:$G_{\alpha o}$ fusion protein was created by mutating the human D_{2S} dopamine receptor stop codon into an alanine residue, followed by two additional alanine residues, constituting a *Not*I restriction site. Similarly, a *Not*I site was inserted at the ATG codon of the rat $G_{\alpha o}$ protein sequence. The fusion was made following *Not*I restriction and ligation. In addition, Cys^{351} of the rat $G_{\alpha o}$ protein was mutated into an Ile (TGT codon into ATT). All the mutations were generated by polymerase chain reaction (PCR), using the Quick Change Site-Directed Kit (Stratagene). The resulting fusion protein sequence was cloned into a pCR3.1 vector (Invitrogen). The construct was fully sequenced to confirm the nucleotide sequence.

2.3. Construction and isolation of recombinant baculovirus

The cDNA fragment containing the D_{2S}:$G_{\alpha o}$ sequence was removed from plasmid pCR3.1 using an *Eco*RI restriction site. The cDNA sequence was then subcloned into the transfer vector pVL1392 (PharMingen) and cotransfected with BaculoGold® DNA in Sf9 cells, using the BaculoGold™ transfection kit (PharMingen). The recombinant baculoviruses were purified and amplified by serial infections of Sf9 cells. The viruses expressing the human G protein β_1 and γ_2 subunits were generously donated by T. Kozasa (University of Illinois, Chicago, IL, USA). All viruses were subjected to PCR to verify their purity and their titres were determined using plaque assays.

2.4. Cell culture

Sf9 cells were grown in suspension in TC-100 medium supplemented with 10% foetal calf serum and 0.1% pluronic F-68®. The cells were maintained at a density of $0.5–2.5\times10^6$ cells/ml and were passaged every 2–3 days. For infections, cells were seeded at a density of 0.6×10^6 cells/ml and infected when they reached log phase growth, i.e. at a density of about 1×10^6 cells/ml. Infections were carried out with D_{2S}:$G_{\alpha o}$ baculovirus alone using a multiplicity of infection (MOI) of 10 or a combination of D_{2S}:$G_{\alpha o}$ with $G\beta_1$ and $G\gamma_2$ baculoviruses. In this latter case the MOIs of the baculoviruses used were 10/7/7 for D_{2S}:$G_{\alpha o}$/$G\beta_1$/$G\gamma_2$, respectively. Cells were harvested 48 h post infection. Where indicated, *Bordetella pertussis* toxin (PTX) was added to the culture medium at a concentration of 100 ng/ml for 18 h prior to cell harvesting.

2.5. Membrane preparation

All operations were carried out at 0–4°C. Cells were collected by centrifugation ($1700\times g$, 10 min, 4°C) and resuspended in 15 ml of buffer (20 mM HEPES, 6 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, pH 7.4). Cell suspensions were then homogenised using an Ultra Turrax at setting 4–5 for 20 s. The homogenate was centrifuged at $1700\times g$ for 10 min and the supernatant was collected and centrifuged at $48\,000\times g$ for 1 h at 4°C. The resulting pellet was resuspended in buffer and stored at −80°C in aliquots of 500 μl. The protein concentration was determined by the method of Lowry et al. [15], using bovine serum albumin as standard.

2.6. Ligand binding assays

[^{3}H]Spiperone saturation binding experiments were performed as described previously [14]. Briefly, eight concentrations of radioligand were used, ranging from approximately 10 pM to 2 nM. The reaction was started by the addition of membrane proteins and incubated for 3 h at 25°C. Reactions were terminated by rapid filtration through Whatman GF/C glass fibre filters using a Brandel cell harvester followed with four washes of 3 ml of ice-cold phosphate-buffered saline (PBS: 140 mM NaCl, 10 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4) and the radioactivity trapped on the filters was determined by liquid scintillation spectrometry. Non-specific binding was defined in the presence of 3 μM (+)-butaclamol. For [^{3}H]spiperone competition binding experiments, a range of concentrations of competing ligand were incubated with a fixed concentration of radioligand (typically 0.45 nM) and the reactions were started and terminated as in [14].

2.7. [^{35}S]GTPγS binding assays

Cell membranes (15–25μg) were incubated in triplicate with 1 μM GDP and increasing concentrations of agonist in a final volume of 0.9 ml buffer (20 mM HEPES, 10 mM $MgCl_2$, 100 mM NaCl, pH 7.4) for 30 min at 30°C. 0.1 ml of [^{35}S]GTPγS was added to a final concentration of 100 pM and the incubation continued for a further 30 min. Basal level of [^{35}S]GTPγS binding was defined as that in the absence of agonist. Incubations were terminated by rapid filtration through Whatman GF/C glass fibre filters using a Brandel cell harvester with four washes of 3 ml of PBS and radioactivity determined as above.

2.8. Analysis of data

Data were analysed using the computer program GraphPad Prism (GraphPad Software). [^{3}H]Spiperone saturation binding curves were fitted best by a one binding site model from which the B_{max} (receptor expression level) and K_d (dissociation constant for [^{3}H]spiperone) were derived. Data from competition experiments were fitted to two binding site and one binding site models, and the best fit was determined using an *F*-test. IC_{50} values of competitors were derived from this analysis and the K_i values (inhibition constants) were calculated using the method of [16]. Concentration–response curves for agonist and inverse agonist effects on [^{35}S]GTPγS binding were analysed by non-linear least squares regression analysis using a sigmoidal concentration/response relationship with a Hill coefficient of 1 and EC_{50} (or IC_{50} for inverse agonists) and E_{max} (maximum effect) values were derived from this analysis. Results are given as mean ± S.E.M. of the indicated number of experiments.

3. Results

3.1. Expression of D_{2S}:$G_{\alpha o}$ fusion protein in Sf9 cells

Expression of the D_{2S}:$G_{\alpha o}$ fusion protein in Sf9 cells was assessed using [^{3}H]spiperone saturation binding assays. As shown in Fig. 1, [^{3}H]spiperone labelled a saturable population of receptors in membranes prepared from Sf9 cells expressing D_{2S}:$G_{\alpha o}$ with or without $G\beta_1\gamma_2$ subunits. The B_{max} obtained amounted to 3290 ± 570 and 2730 ± 230 fmol/mg of protein for the fusion protein alone and the fusion protein co-expressed with $G\beta_1\gamma_2$, respectively. [^{3}H]Spiperone had a high affinity for the D_{2S}:$G_{\alpha o}$ fusion protein expressed alone or with $G\beta_1\gamma_2$ subunits, with pK_d values of 9.83 ± 0.06 (K_d 0.15 nM) and 9.93 ± 0.05 (K_d 0.12 nM), mean ± S.E.M., $n > 7$, for D_{2S}:$G_{\alpha o}$ and D_{2S}:$G_{\alpha o}$ plus $G\beta_1\gamma_2$, respectively. These pK_d values are not significantly different (Student's *t*-test, $P > 0.05$).

3.2. [^{35}S]GTPγS binding assays

The effect of dopamine on the binding of [^{35}S]GTPγS in different preparations was determined, as shown in Fig. 2a.

Table 1
Agonist stimulation of [^{35}S]GTPγS binding to D_{2S}:$G_{\alpha o}$ fusion protein plus $G\beta_1\gamma_2$

Compound	Relative efficacy (% DA)	pEC_{50} ± S.E.M. (EC_{50}, nM)
Dopamine	100	5.43 ± 0.06 (3715)
NPA	154 ± 9	8.25 ± 0.06 (5.62)
Bromocriptine	103 ± 3	8.83 ± 0.14 (1.48)
(+)-3-PPP	69 ± 5	4.91 ± 0.04 (12302)
(−)-3-PPP	17 ± 1	5.72 ± 0.20 (1905)
Quinpirole	81 ± 1	5.74 ± 0.03 (1820)
m-Tyramine	58 ± 1	4.91 ± 0.04 (12302)
p-Tyramine	41 ± 1	3.82 ± 0.06 (151356)
Aripiprazole	10 ± 1	8.84 ± 0.14 (1.45)

A range of concentrations of each agonist was tested for stimulation of [^{35}S]GTPγS binding to the D_{2S}:$G_{\alpha o}$ fusion protein expressed with $G\beta_1\gamma_2$ as described in Section 2 and the concentration–response curves analysed to provide the EC_{50} and the E_{max} (relative to the maximal dopamine effect). The basal [^{35}S]GTPγS binding (in the absence of ligand) amounted to 225 ± 35 fmol/mg protein. Data shown are mean ± S.E.M. from 4–17 experiments.

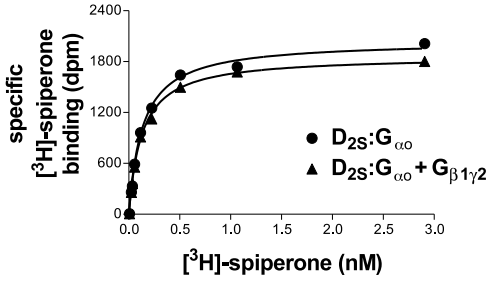

Fig. 1. Expression of D_2 dopamine receptors determined by [^{3}H]spiperone saturation binding. The D_{2S}:$G_{\alpha o}$ fusion protein was expressed alone or with $G\beta_1\gamma_2$ in Sf9 cells. Sf9 membranes expressing the two preparations were subjected to [^{3}H]spiperone saturation binding analysis as described in Section 2. The receptor expression level (B_{max}) and the dissociation constant for [^{3}H]spiperone (K_d) are indicated in the text. Data are representative experiments performed on each membrane preparation and replicated as shown in the text.

Dopamine had no effect on the binding of [^{35}S]GTPγS to membranes prepared from non-infected Sf9 cells. When Sf9 cells expressing the D_{2S}:$G_{\alpha o}$ fusion protein were used, dopamine stimulated the binding of [^{35}S]GTPγS in a concentration-dependent manner, with a maximal effect of 335 ± 66% over basal [^{35}S]GTPγS binding and a pEC_{50} of 5.24 ± 0.06 (EC_{50} 5.75 μM), mean ± S.E.M., $n = 4$. In the presence of the $G\beta_1\gamma_2$ complex, the maximal effect of dopamine was increased to 464 ± 40% and its pEC_{50} was 5.43 ± 0.06 (EC_{50} 3.72 μM), mean ± S.E.M., $n = 17$ (Fig. 2a, Table 1). Treatment of Sf9 cells expressing D_{2S}:$G_{\alpha o}$ fusion protein (plus $G\beta_1\gamma_2$) with PTX (100 ng/ml, 18 h) did not alter the effect of dopamine.

Several D_2 dopamine receptor agonists were tested for their ability to stimulate the binding of [^{35}S]GTPγS to membranes prepared from Sf9 cells expressing the D_{2S}:$G_{\alpha o}$ fusion protein plus $G\beta_1\gamma_2$ (Fig. 2b). All the compounds tested displayed agonist activity, with NPA and bromocriptine appearing as full agonists, and the other ligands, including the putative antipsychotic drug aripiprazole, behaving as partial agonists (Table 1). NPA, bromocriptine and aripiprazole were the most potent agonists, with EC_{50} values three orders of magnitude higher than that of dopamine. Aripiprazole and *S*-(−)-3-PPP exhibited low relative efficacies of 10% and 17%, respectively.

We also analysed the effect of other ligands reported to be antagonists or inverse agonists at D_2 dopamine receptors, on

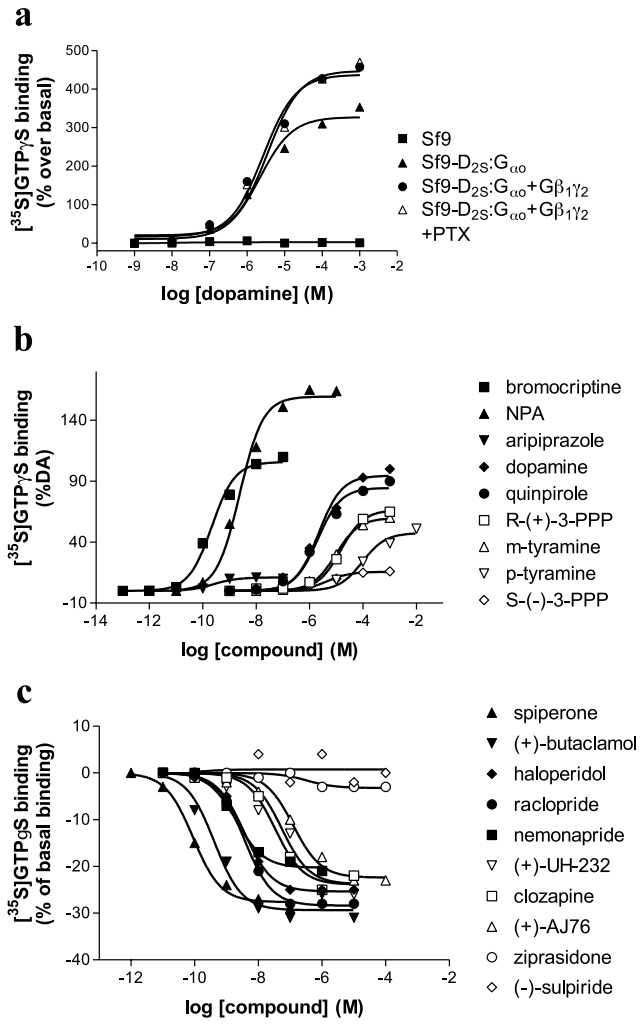

Fig. 2. Modulation of [^{35}S]GTPγS binding in membranes of Sf9 cells expressing D_{2S}:$G_{\alpha o}$ fusion protein. [^{35}S]GTPγS binding was determined as described in Section 2. The basal binding of [^{35}S]GTPγS (in the absence of ligand) amounted to 197 ± 5 fmol/mg protein and 225 ± 35 fmol/mg for D_{2S}:$G_{\alpha o}$ and D_{2S}:$G_{\alpha o}$/$G\beta_1\gamma_2$, respectively. a: Effect of dopamine at different preparations. b: Effect of D_2 receptor agonists at D_{2S}:$G_{\alpha o}$ plus $G\beta_1\gamma_2$. c: Inverse agonist effect of D_2 receptor ligands at D_{2S}:$G_{\alpha o}$ plus $G\beta_1\gamma_2$. Data are from representative experiments replicated as in Tables 1 and 2.

Table 2
Binding affinity and inverse agonist effects of ligands at D_{2S}:$G_{\alpha o}$ plus $G\beta_1\gamma_2$

Compound	pK_i ± S.E.M. (K_i, nM)	pIC_{50} ± S.E.M. (IC_{50}, nM)	E_{max} (% basal)
(+)-Butaclamol	9.66 ± 0.27 (0.22)	9.05 ± 0.37 (0.89)	−31 ± 7
Spiperone	9.82 ± 0.26 (0.15)	9.90 ± 0.27 (0.13)	−30 ± 4
Haloperidol	8.48 ± 0.16 (3.31)	8.59 ± 0.13 (2.57)	−30 ± 3
Raclopride	7.11 ± 0.31 (78)	8.48 ± 0.02 (3.31)	−28 ± 4
Clozapine	6.80 ± 0.15 (158)	7.22 ± 0.14 (60.25)	−28 ± 3
(+)-UH-232	6.80 ± 0.25 (158)	7.60 ± 0.28 (25.11)	−23 ± 5
Nemonapride	8.95 ± 0.02 (1.12)	8.27 ± 0.08 (5.37)	−19 ± 1
(+)-AJ76	5.73 ± 0.07 (1862)	6.53 ± 0.15 (295)	−21 ± 1
Ziprasidone	8.11 ± 0.05 (7.76)	nd	−3 ± 2
(−)-Sulpiride	5.82 ± 0.12 (1514)	na	
(−)-Butaclamol	5.24 ± 0.02 (5754)	nd	

Binding of ligands was determined in competition experiments versus [^{3}H]spiperone binding to membranes expressing D_{2S}:$G_{\alpha o}$ plus $G\beta_1\gamma_2$, as described in Section 2. Competition curves were described best by one binding site models (*F*-test, $P < 0.001$) in all cases and K_i values were derived. A range of concentrations of each compound were also tested for effects on [^{35}S]GTPγS binding to the D_{2S}:$G_{\alpha o}$ fusion protein expressed with $G\beta_1\gamma_2$ as described in Section 2 and the concentration–response curves analysed to provide the IC_{50} and the E_{max} (relative to basal [^{35}S]GTPγS binding) for the inverse agonists. The basal [^{35}S]GTPγS binding (in the absence of ligand) amounted to 225 ± 35 fmol/mg protein. Data shown are mean ± S.E.M. from four to seven independent experiments. na = no activity; nd = not determined.

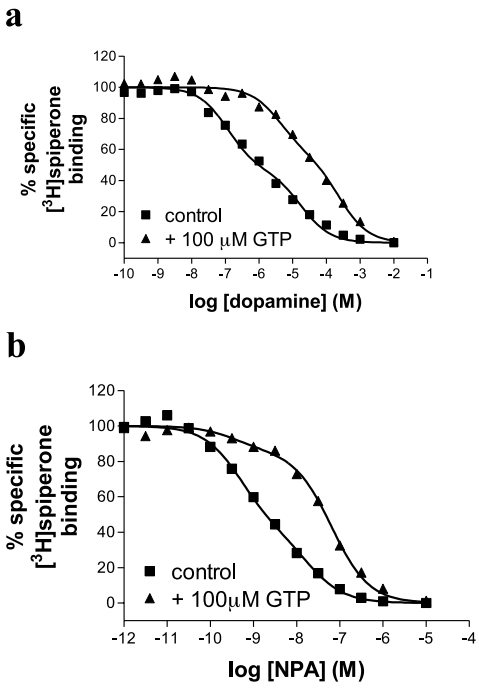

Fig. 3. Agonist binding to membranes of Sf9 cells expressing D_{2S}:$G_{\alpha o}$ plus $G\beta_1\gamma_2$. The binding of dopamine (a) and NPA (b) to D_{2S}:$G_{\alpha o}$ plus $G\beta_1\gamma_2$ was determined in competition versus [^{3}H]spiperone binding in the absence or presence of 100 μM GTP, as described in Section 2. Data shown are representative experiments replicated as in Table 3 and the curves are the best fit curves to two binding site models.

the binding of [^{35}S]GTPγS to membranes prepared from Sf9 cells expressing the D_{2S}:$G_{\alpha o}$ fusion protein plus $G\beta_1\gamma_2$. Most of the ligands tested were able to decrease the basal [^{35}S]GTPγS binding in a concentration-dependent manner exhibiting different maximal inhibitions (E_{max}) and potencies (Fig. 2c, Table 2). Thus, spiperone, (+)-butaclamol and haloperidol showed a maximal inhibition of −30%, whereas nemonapride and (+)-UH-232 were less effective. Spiperone, haloperidol and nemonapride also displayed higher potencies than raclopride, UH-232 and (+)-AJ76. (−)-Sulpiride and ziprasidone, however, did not have any significant effect on the basal [^{35}S]GTPγS binding.

3.3. Competition radioligand binding study

The binding of D_2 dopamine receptor agonists to the D_{2S}:$G_{\alpha o}$ fusion protein expressed in Sf9 cells was investigated in competition experiments versus [^{3}H]spiperone binding. As shown in Fig. 3, in the absence of GTP, competition curves for both dopamine and NPA were fitted best by a two binding site model (*F*-test, $P < 0.001$), with higher and lower affinity binding sites (K_h, K_l). The percentage of higher affinity binding sites found with the two agonists was comparable (~60%, Fig. 3, Table 3). In the presence of 100 μM GTP, the competition curves of both agonists were shifted to the right but a two binding site model still provided the best fit (*F*-test, $P < 0.05$). In the presence of GTP, the proportions of higher affinity binding sites were 40% and 17% for dopamine and NPA, respectively. The K_h as well as the K_l values of dopamine decreased by one order of magnitude, whereas for NPA both values were only marginally affected by the presence of GTP.

A series of D_2 dopamine receptor ligands were also tested for their ability to compete with [^{3}H]spiperone binding to membranes of Sf9 cells expressing D_{2S}:$G_{\alpha o}$ fusion protein plus $G\beta_1\gamma_2$. The competition curves for all the compounds tested were best fitted to a one binding site model (*F*-test, $P < 0.001$, not shown). Spiperone was the most potent competitor, with a pK_i of 9.82, a value comparable to the pK_d of [^{3}H]spiperone (see above). Compounds such as (+)-butaclamol, haloperidol and nemonapride also exhibited high affinity for the D_{2S}:$G_{\alpha o}$/$G\beta_1\gamma_2$, whereas (−)-butaclamol, (+)-UH-232 and (+)-AJ76 exhibited lower affinity for D_{2S}:$G_{\alpha o}$/$G\beta_1\gamma_2$.

4. Discussion

In the present study, we have expressed and characterised a fusion protein between the D_{2S} dopamine receptor and a $G_{\alpha o}$ subunit of G protein. The latter was mutated at position 351, where a cysteine residue was replaced by an isoleucine. Upon expression in Sf9 cells using baculovirus, we demonstrated that the fusion protein was functional, by assessing the ability of dopamine and other D_2 receptor agonists to stimulate the binding of [^{35}S]GTPγS to the D_{2S}:$G_{\alpha o}$ fusion protein. To the best of our knowledge, this is the first study to report a functional coupling of a D_2 dopamine receptor engaged in a fusion with a G_α subunit of G protein.

The D_{2S}:$G_{\alpha o}$ fusion protein expressed in Sf9 cells had a high affinity for [^{3}H]spiperone and the pK_d of this ligand found in the present study is comparable to that found for the receptor expressed in other heterologous systems [14,17]. When a series of ligands were used to compete versus the binding of [^{3}H]spiperone to the D_{2S}:$G_{\alpha o}$ fusion protein, we found a rank order of binding affinity for the different ligands similar to those expected for D_2 receptors. Of particular interest was the stereoselectivity of the binding of butaclamol. Indeed, (−)-butaclamol (the inactive form of the compound) binds to the D_{2S}:$G_{\alpha o}$ fusion protein with three orders of magnitude lower affinity compared to (+)-butaclamol (the active form of the compound). These results show that the D_{2S}

Table 3
Agonist binding to membranes of Sf9 cells expressing D_{2S}:$G_{\alpha o}$ plus $G\beta_1\gamma_2$

	Dopamine		NPA	
	Control	+100 μM GTP	Control	+100 μM GTP
% R_h	59 ± 7	41 ± 2	58 ± 3	17 ± 2
pK_h ± S.E.M. (K_h, nM)	7.32 ± 0.36 (48)	5.89 ± 0.05 (1288)	10.13 ± 0.13 (0.07)	9.92 ± 0.12 (0.12)
pK_l ± S.E.M. (K_l, nM)	5.25 ± 0.27 (5623)	4.18 ± 0.04 (66 069)	8.32 ± 0.08 (5)	7.75 ± 0.01 (18)

Binding of dopamine and NPA was determined in competition experiments versus [^{3}H]spiperone binding to membranes expressing D_{2S}:$G_{\alpha o}$ plus $G\beta_1\gamma_2$, as described in Section 2. Competition curves were described best by two binding site models (*F*-test, $P < 0.002$) in all cases and values for the dissociation constant at the higher (K_h) and lower (K_l) affinity sites and the % higher affinity sites (% R_h) are given. Data are mean ± S.E.M. from four independent experiments.

receptor engaged in a fusion with the $G_{\alpha o}$ subunit of G protein is being expressed with fidelity in Sf9 cells.

Several agonists were then tested for their ability to stimulate [^{35}S]GTPγS binding to membranes expressing D_{2S}:$G_{\alpha o}$ fusion protein. Dopamine induced a concentration-dependent stimulation of [^{35}S]GTPγS binding to membranes expressing the D_{2S}:$G_{\alpha o}$ fusion protein, an effect which was absent in non-infected Sf9 cells (Fig. 2). Interestingly, when the D_{2S}:$G_{\alpha o}$ fusion protein was co-expressed with $G\beta_1\gamma_2$, the maximal effect of dopamine was significantly increased. Thus, the presence of $G\beta_1\gamma_2$ may modulate the information transfer between the receptor and its cognate G protein, as reported by others for a β_2-adrenergic receptor/$G_{\alpha s}$ fusion protein [18]. We, therefore, decided to use the preparation expressing D_{2S}:$G_{\alpha o}$ fusion protein with $G\beta_1\gamma_2$ for further characterisation. As some receptors engaged in a fusion protein with a cognate G protein subunit have been shown to interact with other endogenous G proteins [19–21], we sought to verify the specific activation of the $G_{\alpha o}$ subunit involved in the fusion protein with the D_{2S} receptor by treating the cells with PTX. In fact, mutation of the G_α subunit of the G proteins belonging to the $G_{i/o}$ family at position 351 has been shown to induce a resistance of the corresponding G protein to the ADP-ribosylation action of PTX. These mutations have been extensively used in the literature [5,6]. In the present study, we found similar effects for dopamine for the stimulation of [^{35}S]GTPγS binding in the presence and in the absence of PTX. This suggests that the D_{2S} dopamine receptor engaged in the fusion protein is coupling exclusively to the $G_{\alpha o}$ to which it is fused.

All the compounds tested showed agonist activity in the preparation expressing D_{2S}:$G_{\alpha o}$ fusion protein with $G\beta_1\gamma_2$, with relative efficacies ranging from 10% to up to 150% compared to dopamine. Thus, compounds such as NPA and bromocriptine behaved as full agonists compared to dopamine, and all the other compounds behaved as partial agonists, including aripiprazole (Fig. 2b, Table 1). The potency of the compounds found in the present study was somewhat lower than potencies reported for this receptor in other heterologous systems (see for example [17]). This may reflect the differences in the expression system (CHO versus Sf9 cells). However, the rank order observed for the potency is that expected for D_2 receptor activation, with NPA and bromocriptine being the more potent compounds and *p*-tyramine showing the lowest potency.

A range of ligands were also tested for their ability to modulate the binding of [^{35}S]GTPγS to preparations expressing D_{2S}:$G_{\alpha o}$ fusion protein with $G\beta_1\gamma_2$. These included antipsychotic drugs such as clozapine and haloperidol. Interestingly, most of the compounds tested behaved as inverse agonists at the D_{2S}:$G_{\alpha o}$ fusion protein. Moreover, different potencies could be observed for the different compounds tested, with (+)-butaclamol, spiperone and nemonapride appearing as the most potent inverse agonists. The maximal inhibition levels for these compounds were also variable, with nemonapride and (+)-AJ76 showing the lower effect. Surprisingly, (+)-UH-232 behaved as an inverse agonist in the present study. This compound has been described as a neutral antagonist at the D_2 receptor in some reports [22]. Several studies, however, reported partial agonist activity for (+)-UH-232 at the D_2 dopamine receptor [23–25]. Such a different range of efficacy observed for the same ligand acting at one receptor might reflect a conformation of the receptor induced by a ligand, which differentially couples to G proteins. In fact other studies have used CHO or COS-7 cells expressing the D_2 receptor [22–25]. Indeed the CHO cells express high levels of G_i proteins (G_{i2}, G_{i3}) and little G_o [26,27]. Thus, (+)-UH-232 might behave as an inverse agonist when the receptor is interacting exclusively with G_o proteins. However, the interaction of the D_2 receptors with a different set of G proteins (in particular G_i proteins) may lead to different effects observed with (+)-UH-232. Similarly, we have reported recently the regulation of G protein selectivity by agonists at rat and human D_{2L} receptors [13,14]. It is noteworthy that compounds such as (−)-sulpiride and ziprasidone do not have significant effects at the D_{2S}:$G_{\alpha o}$ fusion protein, suggesting that in the presence of G_o, these compounds behave as pure antagonists at the D_2 receptor. This may apply to the G_q G protein as well, as in a recent study using a chimeric D_2/α_{1B} receptor, ziprasidone behaved as a silent antagonist even though the constitutive activity of the dopamine D_2 receptor was increased [25].

Although a fusion protein would be expected to exhibit agonist binding curves with Hill coefficients close to 1, several studies have reported higher and lower agonist affinity states (see for example [3,28] and [5] for a review). In the present study, when dopamine and NPA were used to compete with the binding of [^{3}H]spiperone to preparations expressing D_{2S}:$G_{\alpha o}/G\beta_1\gamma_2$, the competition data were fitted best by two binding site models. Moreover, the presence of 100 μM GTP failed to convert the binding curves to a single (low) affinity state. Furthermore, the percentage of higher affinity binding sites of NPA was dramatically decreased, whereas that of dopamine was only marginally affected by the presence of GTP. Conversely, the affinities of dopamine for both higher and lower affinity states of the receptor were significantly decreased, whereas those of NPA were marginally affected by the presence of GTP. This different sensitivity of dopamine and NPA to GTP may be related to the fusion process. Indeed, spatial constrains may exist due to the physical proximity and tethering of the carboxy-terminus of the D_{2S} receptor and the NH_2 terminus of the $G_{\alpha o}$. It is also possible that the mutation of the G_α subunit ($Cys^{351}Ile$) increases the interaction of the G protein with the receptor as reported by others (see [5] for a review). Furthermore, different conformations of the same receptor induced by the binding of the two agonists, as proposed recently [29] and exemplified in several studies [13,14], could also explain these observations.

In conclusion, this study has described for the first time the properties of a fusion protein involving a D_2 dopamine receptor and its cognate G protein. Our results show that such a construct expressed in Sf9 cells exhibits characteristics similar to those observed in other heterologous systems expressing the D_2 receptor. Interestingly, such a system can be used to characterise both the binding properties and the activity of a range of ligands (agonists, neutral antagonists, inverse agonists) at the D_2 dopamine receptor. The data obtained in this study, where the D_2 receptor is forced to interact with G_o, suggest that some antipsychotic drugs exhibit different relative efficacies depending on the particular receptor/G protein combination. The data may be of additional relevance as in brain the principal G protein interacting with the D_2 receptor is G_o [30]. Further fusion constructs between the D_2 receptor and other G proteins may help to clarify the activities of antipsychotic drugs at the D_2 dopamine receptors.

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