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Protean Agonism at the Dopamine D_2 Receptor: (*S*)-3-(3-Hydroxyphenyl)-*N*-propylpiperidine Is an Agonist for Activation of G_{o1} but an Antagonist/Inverse Agonist for G_{i1}, G_{i2}, and G_{i3}

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ABSTRACT

A range of ligands displayed agonism at the long isoform of the human dopamine D_2 receptor, whether using receptor-G protein fusions or membranes of cells in which pertussis toxin-resistant mutants of individual $G\alpha_i$-family G proteins could be expressed in an inducible fashion. Varying degrees of efficacy were observed for individual ligands as monitored by their capacity to load [^{35}S]GTPγS onto each of $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, and $G\alpha_{o1}$. By contrast, (*S*)-(−)-3-(3-hydroxyphenyl)-*N*-propylpiperidine was a partial agonist when $G\alpha_{o1}$ was the target G protein but an antagonist/inverse agonist at $G\alpha_{i1}$, $G\alpha_{i2}$, and $G\alpha_{i3}$. In ligand binding assays, dopamine identified both high- and low-affinity states at each of the dopamine D_2 receptor-G protein fusion proteins, and the high-affinity state was eliminated by guanine nucleotide. (*S*)-(−)-3-(3-Hydroxyphenyl)-*N*-propylpiperidine bound to an apparent single state of the constructs in which the D_2 receptor was fused to $G\alpha_{i1}$, $G\alpha_{i2}$, or $G\alpha_{i3}$. However, it bound to distinct high- and low-affinity states of the D_2 receptor-$G\alpha_{o1}$ fusion, with the high-affinity state being eliminated by guanine nucleotide. Likewise, although dopamine identified guanine nucleotide-sensitive high-affinity states of the D_2 receptor when expression of pertussis toxin-resistant forms of each of $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, and $G\alpha_{o1}$ was induced, (*S*)-(−)-3-(3-hydroxyphenyl)-*N*-propylpiperidine identified a high-affinity site only in the presence of $G\alpha_{o1}$. *p*-Tyramine displayed a protean ligand profile similar to that of (*S*)-(−)-3-(3-hydroxyphenyl)-*N*-propylpiperidine but with lower potency. These results demonstrate (*S*)-(−)-3-(3-hydroxyphenyl)-*N*-propylpiperidine to be a protean agonist at the D_2 receptor and may explain in vivo actions of this ligand.

A large number of G protein-coupled receptors (GPCRs) are able to generate a variety of intracellular signals, and for those with a rich pharmacology of synthetic small-molecule ligands, it has often been possible to observe differential pharmacology for individual end points (Perez and Karnik, 2005). This has resulted in an appreciation that different ligands may stabilize distinct conformational states of GPCRs (Kenakin, 2001; Perez and Karnik, 2005) and in an expansion of the simple "active" or "inactive" "two-state" model (Leff, 1996) of GPCR function into "three-state" models (Leff et al., 1997) and subsequent chemical and physical considerations of GPCRs that allow the potential for an essentially unlimited number of states (Milligan and IJzerman, 2000; Vauquelin and Van Liefde, 2005). Although GPCRs are defined by their capacity to activate heterotrimeric G proteins, a number of ligand-induced signals seem not to require G protein interactions (Wei et al., 2003; Gesty-Palmer et al., 2006). In the case of the β_2-adrenoceptor, for example, such separation of signal transduction has resulted in the identification of ligands that can be defined as inverse agonists for their effects on adenylyl cyclase activity but as agonists for their capacity to stimulate phosphorylation of the extracellular signal-regulated kinases 1 and 2 mitogen-activated protein kinases (Azzi et al., 2003; Galandrin and Bouvier, 2006). Ligands that display either positive or negative efficacy when assessed in different assays or in different experimental conditions have been described as "protean" ligands (Kenakin, 2001) and have been of particular value in defining the ability of GPCRs to adopt different conformational states. Many GPCRs are also able to couple to a number of different G

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ABBREVIATIONS: GPCR, G protein-coupled receptor; 7-OH-DPAT, *R*-(+)-7-hydroxy-2-dipropylaminotetralin hydrobromide; D2L, long isoform of the human dopamine D_2 receptor; [^{35}S]GTPγS, guanosine 5′-*O*-(3-[^{35}S]thio)triphosphate; NPA, *R*-(−)-10,11-dihydroxy-*N*-*n*-propylnorapomorphine; *R*-(+)-3-PPP, (*R*)-(+)-3-(3-hydroxyphenyl)-*N*-propylpiperidine; *S*-(−)-3-PPP, (*S*)-(−)-3-(3-hydroxyphenyl)-*N*-propylpiperidine; PCR, polymerase chain reaction; HEK, human embryonic kidney.

proteins, and differences in agonist pharmacology to regulate signals via different G proteins are described frequently as "agonist-directed trafficking" (Kenakin, 1995). In many of these studies, observations have concentrated predominantly on measuring varying efficacy of ligands to regulate the production of two separate second messengers rather than directly measuring differential activation of two individual G proteins (Berg et al., 1998).

The dopamine D_2 receptor has been one of the most studied monoaminergic GPCRs, not least because of the affinity of a wide range of antipsychotic agents for this receptor (Akam and Strange, 2004). As with a series of GPCRs that interact with members of the pertussis toxin-sensitive subgroup of G proteins, this receptor is able to initiate signals via each of $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, and $G\alpha_{o1}$ (Gazi et al., 2003). However, ligand pharmacology can be influenced greatly by the ratio of GPCR to G protein expression (Milligan, 2000), and this can be difficult to define in cells, particularly in studies designed to compare activation and function of different G proteins. One means to overcome this issue is to use GPCR-G protein fusions that ensure a fixed 1:1 stoichiometry of GPCR and G protein (Milligan et al., 2004). Because pertussis toxin-sensitive G proteins are endogenously expressed by all cells, we have also used previously variants of each of $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, and $G\alpha_{o1}$ that have been rendered insensitive to the ADP-ribosyltransferase activity of pertussis toxin by mutation of the cysteine, four amino acids from the C terminus that is the site of modification, to isoleucine (Bahia et al., 1998; Wise et al., 1999).

By using both fusions of the long isoform of the human dopamine D_2 (D2L) receptor with pertussis toxin-resistant cysteine-isoleucine variants of each of $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, and $G\alpha_{o1}$ and cell lines stably expressing the D2L receptor in which varying amounts of each G protein can be expressed in an entirely tetracycline-dependent manner, we now demonstrate that both (*S*)-(−)-3-(3-hydroxyphenyl)-*N*-propylpiperidine [*S*-(−)-3-PPP] and *p*-tyramine are protean ligands at the D2L receptor, being agonists for activation of $G\alpha_{o1}$ but antagonists/inverse agonists at $G\alpha_{i1}$, $G\alpha_{i2}$, and $G\alpha_{i3}$. Such observations provide further evidence for the concept that the dopamine D_2 receptor can exist in multiple conformational states and indicate that it is possible to selectively control the nature of signals generated by D_2 receptor "agonists." Because individual pertussis toxin-sensitive G proteins are expressed differentially pre- and postsynaptically (Aoki et al., 1992), these observations may be relevant to the reported in vivo actions of *S*-(−)-3-PPP (Arnt et al., 1983; Hjorth et al., 1983).

Materials and Methods

Materials. [^{3}H]Spiperone (65–140 Ci/mmol) was from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK), and guanosine 5′-*O*-(3-[^{35}S]thio)triphosphate ([^{35}S]GTPγS; 1250 Ci/mmol) was from PerkinElmer Life and Analytical Sciences (Boston, MA). (+)-Butaclamol, dopamine, (−)-quinpirole, *m*-tyramine, *p*-tyramine, *S*-(−)-PPP, (*R*)-(+)-3-(3-hydroxyphenyl)-*N*-propylpiperidine [*R*-(+)-3-PPP], (*R*)-(−)-10,11-dihydroxy-*N*-*n*-propylnorapomorphine (NPA), (*R*)-(+)-7-hydroxy-DPAT hydrobromide (7-OH-DPAT), and GTPγS were purchased from Sigma (Gillingham, Dorset, UK). Spiperone hydrochloride was from Tocris (Bristol, UK). Oligonucleotides were from ThermoElectron (Ulm, Germany), and all materials for tissue culture were from Invitrogen (Paisley, UK). All other reagents were obtained as indicated.

D_2 Dopamine Receptor Subcloning into pcDNA3. D2L was initially in the vector pDEST12.2. D2L cDNA was amplified by PCR using the following primers: sense, AAA AGA ATC CGC CAC CAT GGA TCC ACT GAA TCT GTC C; antisense, AAA ACT CGA GTC AGC AGT GGA GGA TCT TCA GGA AGG. Underlined bases indicate the restriction sites EcoRI (sense) and XhoI (antisense). The resulting PCR fragment was digested with EcoR I and XhoI and inserted into pcDNA3.

Construction of the Myc-D2L-G-Protein α Subunit Fusion Proteins. Pertussis toxin-resistant α_{2A}-adrenoceptor-G-protein fusion proteins had been prepared as described previously (Wise and Milligan, 1997; Cavalli et al., 2000). In brief, Cys^{351} of rat $G\alpha_{i1}$, $G\alpha_{i3}$, and $G\alpha_{o1}$ (Cys^{352} in $G\alpha_{i2}$) was mutated to isoleucine by site-directed mutagenesis and then used to create the α_{2A}-adrenoceptor-Gα fusion proteins using the porcine α_{2A}-adrenoceptor in pcDNA3. These constructs were cloned into pcDNA3 using a created 5′ KpnI site and 3′ EcoRI site with a NcoI site between receptor and G-protein α subunit cDNAs. To create D2L:G protein α subunit proteins, the first step was to remove the NcoI site from within the D2L cDNA by site-directed mutagenesis using a QuikChange Mutagenesis kit (Stratagene, La Jolla, CA) and the following primers: sense, 5′-CC GAC CCG TCC CAT CA**T** GGT CTC CAC AG-3′; antisense, 5′-CT GTG GAG ACC ATG **A**TG GGA CGG GTC GG-3′. Boldface letters indicate altered bases. The PCR product was then digested with DpnI and transformed into bacteria. In a similar manner, NcoI sites were removed from both the $G\alpha_{i1}$ and $G\alpha_{o1}$ cDNAs in the respective α_{2A}-adrenoceptor-Gα fusion protein cDNAs using the following primers: $G\alpha_{i1}$ sense, 5′-TT GCC ATC ATT AGA GCG ATG GGG AGA TTG AAA ATC G-3′; antisense, 5′-C GAT TTT CAA TCT CCC CAT CGC TCT AAT GAT GGC AA-3′; and $G\alpha_{o1}$ sense, 5′-CC ATT GTG CGG GCG ATG GAT ACT CTG GG-3′; antisense, 5′-CC CAG AGT ATC CAT CGC CCG CAC AAT GG-3′.

Myc-D2L (NcoI-) was amplified by PCR using the following primers: sense, 5′- AGA ACG GGG TAC CTT ATG **GAA CAA CAA AAA CTT ATT TCT GAA GAA GAT CTG** GAT CCA CTG AAT CTG TCC TGG TAT GAT G-3′; antisense, 5′-AAAAAAAACCAT GGAGT-GGAGGATCTTCAGGAAGGC-3′. Underlined bases indicate introduced restriction sites (sense, KpnI; antisense, NcoI), bases in boldface type indicate introduced N-terminal Myc tag. The PCR fragment was digested using KpnI and NcoI.

The α_{2A}-adrenoceptor-Gα fusion proteins (NcoI-) were excised from pcDNA3 using KpnI and EcoRI, digested with NcoI, and the Gα subunit cDNA was purified. The $G\alpha_i$ subunit cDNAs were then cloned into pcDNA3 with the Flag-D2L PCR fragment to create the four D2L:G-protein α subunit fusion proteins.

Flp-In Constructs. Previously, pertussis toxin-resistant mutants of rat $G\alpha_{i1}$, $G\alpha_{i3}$, and $G\alpha_{o1}$ were created by mutation of Cys^{351} to isoleucine (Cys^{352} for $G\alpha_{i2}$) by site-directed mutagenesis. These were cloned into pcDNA3. cDNAs were excised using KpnI and ApaI ($G\alpha_{i1-3}$) or ApaI ($G\alpha_{o1}$) and subcloned into the pcDNA5/FRT/TO vector (Invitrogen).

Cell Culture and Transfection. HEK293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 0.292 g/l L-glutamine and 10% (v/v) newborn calf serum at 37°C in a 5% CO_2 humidified atmosphere. Cells were grown to 60 to 80% confluence before transient transfection. Transfection was performed using Lipofectamine transfection reagent (Invitrogen) according to the manufacturer's instructions.

Generation of Stable Flp-In T-REx HEK293 Cells. To generate Flp-In T-REx HEK293 cells able to inducibly express the G protein α subunit of interest, the cells were transfected with a mixture containing the desired G protein α subunit cDNA in the pcDNA5/FRT/TO vector and pOG44 vectors (1:9) using Lipofectamine according to the manufacturer's instructions. Cell maintenance and selection were as described previously (Milasta et al., 2006). Clones were screened for G protein expression by Western

blotting. To constitutively stably coexpress the D2L receptor in inducible cell lines, the appropriate cells were further transfected with the D2L receptor cDNA in pcDNA3 as described above, and resistant cells were selected in the presence of 1 mg/ml G418. Resistant clones were screened for receptor expression using specific [^{3}H]spiperone binding. Cells were treated with 1 μg/ml doxycycline 24 to 48 h before assay to induce the expression of G protein α subunits cloned into the Flp-In locus.

Membrane Preparation. Cells were collected by centrifugation (1700*g*, 5 min, 4°C) frozen at −80°C for at least 1 h and resuspended in 15 ml of buffer (10 mM Tris and 0.1 mM EDTA, pH 7.4). Cell suspensions were then homogenized using an Ultra Turrax for 3 × 20 s. The homogenate was centrifuged at 1700*g* for 10 min, and the supernatant was collected and centrifuged at 48,000*g* for 45 min at 4°C. The resulting pellet was resuspended in buffer and stored at −80°C in aliquots of 1 ml.

Saturation Binding Assays Using [^{3}H]Spiperone. Cell membranes (10 μg of protein) were incubated in triplicate with [^{3}H]spiperone (0.001–2 nM) in a total volume of 1 ml of buffer (20 mM HEPES, 6 mM $MgCl_2$, 1 mM EDTA, and 1 mM EGTA, pH 7.4). Nonspecific binding was determined by the inclusion of 10 μM (+)-butaclamol. The reaction was initiated by the addition of membranes, and the tubes were incubated at 25°C for 3 h. The reaction was terminated by rapid filtration using a Brandel cell harvester with three 5-ml washes of ice-cold phosphate-buffered saline (140 mM NaCl, 10 mM KCl, 1.5 mM KH_2PO_4, and 8 mM Na_2HPO_4). The filters were soaked in 3 ml of scintillation fluid, and radioactivity present was determined by liquid scintillation spectrometry.

Agonist Competition versus [^{3}H]Spiperone Binding. Cell membranes (10 μg of protein) were incubated with 0.05 nM [^{3}H]spiperone and various concentrations of agonists, in triplicate, in a final volume of 1 ml of buffer (20 mM HEPES, 6 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, and 40 μM ascorbic acid, pH 7.4). Nonspecific binding was determined by the inclusion of 10 μM (+)-butaclamol. The reactions were initiated, incubated, and terminated as described above. The effect of guanine nucleotides on dopamine binding was

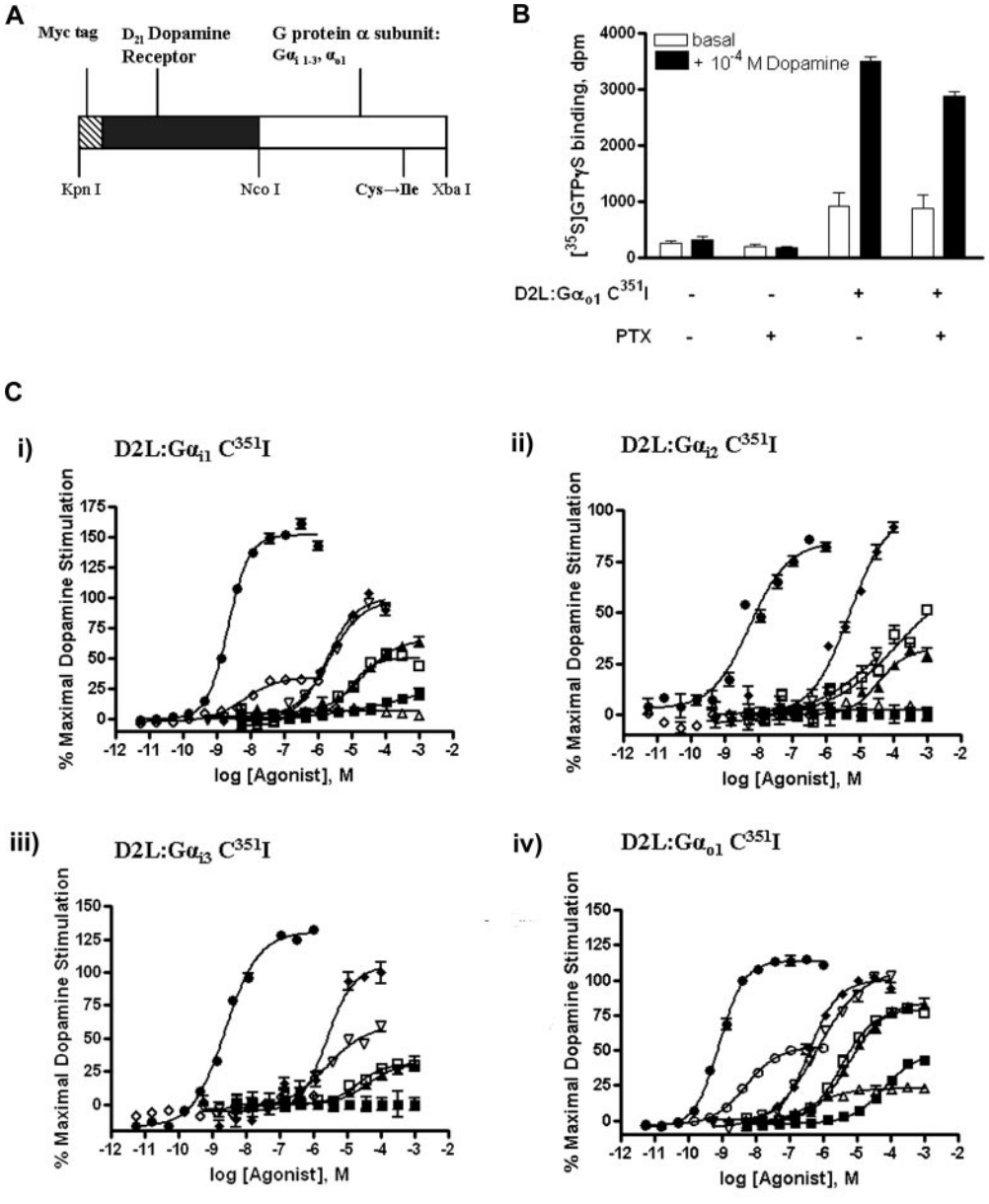

Fig. 1. G protein fusion proteins identify agonists and protean ligands at the D2L receptor. A, a series of fusion proteins was generated by linking the cysteine-isoleucine (C-I) mutant, pertussis toxin-insensitive variants of $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, and $G\alpha_{o1}$ in frame with the C-terminal tail of the human D2L receptor. B, HEK293 cells were mock-transfected or transfected to express D2L-$G\alpha_{o1}$ transiently and treated (+) or not (−) with pertussis toxin (PTX) (25 ng/ml, 24 h). Membranes of these cells were used in [^{35}S]GTPγS binding studies in the absence (□) or presence (■) of 100 μM dopamine. C, each of the fusion proteins D2L-$G\alpha_{i1}$ (i), D2L-$G\alpha_{i2}$ (ii), D2L-$G\alpha_{i3}$ (iii), and D2L-$G\alpha_{o1}$ (iv) was expressed transiently in HEK293 cells. After pertussis toxin treatment (25 ng/ml, 24 h), cells were harvested, membranes were generated, and [^{35}S]GTPγS binding studies were performed in the absence and presence of varying concentrations of a variety of ligands [dopamine (♦), *m*-tyramine (□), *p*-tyramine (■), *R*-(+)-3-PPP (▲), *S*-(−)-3PPP (△), quinpirole (▽), NPA (●), and 7-OH DPAT (◊)] reported to have affinity and efficacy at dopamine D_2 receptors. Data are representative, and full details are provided in Table 2.

assessed by the addition of 100 mM NaCl and 100 μM GTP to the buffer.

[^{35}S]GTPγS Binding Assays. Cell membranes (10 μg) were incubated in 900 μl of buffer (20 mM HEPES, 100 mM NaCl, 6 mM $MgCl_2$, and 40 μM ascorbic acid, pH 7.4) containing 10 μM GDP and various concentrations of ligands. All experiments were performed in triplicate. The reaction was initiated by the addition of cell membranes and incubated at 30°C for 30 min. A 100-μl volume of [^{35}S]GTPγS (0.1 nM final concentration) was then added, and the incubation continued for a further 30 min. The reaction was terminated by rapid filtration with a Brandel cell harvester and three 4-ml washes with ice-cold phosphate-buffered saline. Radioactivity was determined as described for saturation analysis. For antagonist dose-response assays, an EC_{50} concentration of dopamine was added along with various concentrations of antagonist.

[^{35}S]GTPγS Binding Assay: Agonist Stimulation of [^{35}S]GTPγS Binding by Fusion Proteins. [^{35}S]GTPγS binding assays were performed at room temperature in 384-well format. Membranes (10 μg/point) were diluted to 0.4 mg/ml in assay buffer (20 mM HEPES, 100 mM NaCl, and 10 mM $MgCl_2$, pH 7.4) supplemented with saponin (10 mg/l) and preincubated with 10 μM GDP and wheat germ agglutinin SPA beads (GE Healthcare) (0.5 mg) and incubated at room temperature for 45 min with agitation. Various concentrations of D_2 dopamine receptor agonists were added, followed by [^{35}S]GTPγS (1170 Ci/mmol; GE Healthcare) at 0.3 nM (total volume of 46 μl), and binding was allowed to proceed at room temperature for 4 hours. Bound [^{35}S]GTPγS was determined by scintillation counting on a ViewLux ultraHTS Microplate Imager (PerkinElmer).

Data Analysis. Data were analyzed using Prism software (GraphPad Software Inc., San Diego, CA).

Results

Studies with Dopamine D_2 Receptor-G Protein Fusions. It has been established previously that the dopamine D_2 receptor is able to interact with and activate each of the pertussis toxin-sensitive G proteins $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, and $G\alpha_{o1}$ (Gazi et al., 2003). Because particularly $G\alpha_{i2}$ and $G\alpha_{i3}$ are expressed endogenously by virtually all cells and we wished to examine potential variations in the ability of ligands at the D2L receptor to activate the different G proteins, cysteine to isoleucine, pertussis toxin-insensitive mutants of the α subunit of each of G_{i1}, G_{i2}, G_{i3}, and G_{o1} (Wise et al., 1999) were linked in-frame with the C-terminal tail of the human D2L (Fig. 1A). This ensured that the stoichiometry of receptor to G protein would be identical for each G protein to be studied and that the relative cellular distribution and orientation of receptor and G protein would be uniform. Each fusion protein was expressed transiently in HEK293 cells. After pertussis toxin treatment (25 ng/ml, 24 h) of these cells to cause ADP-ribosylation of the endogenously expressed forms of "$G\alpha_i$" and membrane preparation, saturation binding assays using [^{3}H]spiperone indicated each fusion protein to be expressed to similar levels and to bind this ligand with similar and high affinity (Table 1). Membranes of HEK293 cells mock-transfected or transfected to express D2L-Cys^{351}Ile $G\alpha_{o1}$ and treated or not with pertussis toxin were used in [^{35}S]GTPγS binding studies. In the absence of D2L-Cys^{351}Ile $G\alpha_{o1}$, binding of the nucleotide was low, essentially unaffected by pertussis toxin treatment, and not modulated by the addition of dopamine (Fig. 1B). With expression of D2L-Cys^{351}Ile $G\alpha_{o1}$, [^{35}S]GTPγS binding in the absence of dopamine was increased, and this level was increased substantially further in the presence of dopamine (Fig. 1B). Pertussis toxin treatment produced a small decline in dopamine-stimulated [^{35}S]GTPγS binding, consistent with the D2L receptor within the fusion being able to access endogenously expressed G proteins, but after pertussis toxin treatment, the elevation of [^{35}S]GTPγS binding by dopamine remained robust (Fig. 1B), indicating direct activation of the fused G protein by the D2L receptor. Equivalent [^{35}S]GTPγS binding assays demonstrated all of the fusion proteins to be activated in a pertussis toxin-insensitive manner by dopamine, NPA, quinpirole, *m*-tyramine, and R-(+)-3-PPP (Fig. 1C), although compared with dopamine, only NPA was a full agonist at each construct, and potency of the individual ligands varied significantly at the various fusion constructs (Table 2). With the exception of NPA and 7-OH DPAT, potency of the ligands was greatest for D2L-Cys^{351}Ile $G\alpha_{o1}$ (Table 2), whereas the potency of quinpirole was particularly low at D2L-Cys^{352}Ile $G\alpha_{i2}$ (Table 2). Unlike the ligands mentioned above, although both *p*-tyramine and *S*-(−)-3-PPP dis-

TABLE 1

[^{3}H]Spiperone binds with similar and high affinity to various D2L receptor-G protein fusions

Individual D2L-G protein fusions were expressed transiently in HEK293 cells. Saturation [^{3}H]spiperone ligand binding studies were performed on membrane preparations as detailed under *Materials and Methods*. Data represent means (±S.E.M.) of studies performed on membranes prepared from three individual transfections.

Receptor:G Protein Fusion	K_d	B_{max}
	nM	*fmol/mg*
D2L-$G\alpha_{i1}$	0.049 (0.003)	1500 (49)
D2L-$G\alpha_{i2}$	0.051 (0.003)	1415 (24)
D2L-$G\alpha_{i3}$	0.057 (0.002)	1976 (14)
D2L-$G\alpha_{o1}$	0.057 (0.006)	1508 (43)

TABLE 2

The potency and efficacy of ligands at D2L receptor-G protein fusions

[^{35}S]GTPγS binding studies were performed as described under *Materials and Methods* on membranes of HEK293 cells transfected to transiently express each of the D2L-G protein fusions. Estimates of pEC_{50} values (±S.E.M.) for each ligand and agonist efficacy measurements relative to dopamine (±S.E.M.) each are provided.

	$D_{2l}G\alpha_{i1}$		$D_{2l}G\alpha_{i2}$		$D_{2l}G\alpha_{i3}$		$D_{2l}G\alpha_{o1}$	
	pEC_{50}	E_{max}	pEC_{50}	E_{max}	pEC_{50}	E_{max}	pEC_{50}	E_{max}
		%DA		*%DA*		*%DA*		*%DA*
Dopamine	5.63 (0.05)	100	5.25 (0.16)	100	4.92 (0.15)	100	6.15 (0.15)	100
m-Tyramine	4.81 (0.14)	50 (2)	4.92 (0.32)	40 (13)	4.97 (0.30)	34 (2)	5.38 (0.03)	74 (3)
p-Tyramine	N.A.		N.A.		N.A.		3.85 (0.20)	53 (4)
R-(+)-3-PPP	4.77 (0.06)	61 (2)	4.67 (0.30)	33 (2)	4.59 (0.23)	41 (10)	5.21 (0.02)	82 (1)
S-(−)-3-PPP	N.A.		N.A.		N.A.		6.25 (0.09)	21 (2)
Quinpirole	5.53 (0.06)	99 (1)	4.72 (0.76)	59 (27)	5.62 (0.42)	55 (15)	6.12 (0.03)	100 (4)
NPA	7.84 (0.45)	143 (6)	7.64 (0.23)	96 (18)	7.48 (0.31)	101 (17)	7.86 (0.58)	109 (5)
7-OH-DPAT	8.12 (0.13)	34 (1)	N.A.		7.83 (0.30)	21 (2)	7.99 (0.21)	51 (1)

N.A., no detectable stimulation; %DA, percentage of maximum response to dopamine.

played agonism at the D2L-Cys^{351}Ile Gα_{o1} fusion (Table 2), they did not enhance [^{35}S]GTPγS binding to any of the other fusions. Although *p*-tyramine displayed greater agonist efficacy than *S*-(−)-3-PPP at the D2L-Cys^{351}Ile Gα_{o1} fusion, subsequent detailed studies used *S*-(−)-3-PPP because its potency as an agonist at D2L-Cys^{351}Ile Gα_{o1} was 300-fold greater than *p*-tyramine (Table 2).

The ability of dopamine to compete with [^{3}H]spiperone to bind to the various D2L-G protein fusions (Fig. 2) was best fit by a two-site model in which between 30 and 50% of the sites displayed higher affinity (pK_h = 7.1–7.7) and the remainder lower affinity (pK_l = 5.6–5.8) for dopamine (Table 3). The presence of 100 μM GTP in such assays resulted in this competition becoming monophasic (pK = 5.5–5.9) in each case (Table 3). By contrast, the ability of *S*-(−)-3-PPP to compete with [^{3}H]spiperone (Fig. 2) was monophasic in the absence of GTP and essentially unaffected by the presence of GTP (pK = 6.2–6.5) for each of the fusions except for D2L-Cys^{351}Ile Gα_{o1} in which a biphasic competition curve (pK_h = 8.4, pK_l = 6.2) was converted to a monophasic curve (pK = 6.3) in the presence of GTP (Table 3).

To explore the details of this apparent protean (Kenakin, 2001) characteristic of *S*-(−)-3-PPP at the D2L, we compared effects at D2L-Cys^{351}Ile Gα_{o1} and D2L-Cys^{352}Ile Gα_{i2} because Gα_{i2} and Gα_{o1} have the lowest sequence identity among the four G proteins studied, and the observed variation in ligand potency for activation of Gα_{o1} and the other G proteins was most consistent for Gα_{i2}. Increasing concentra-

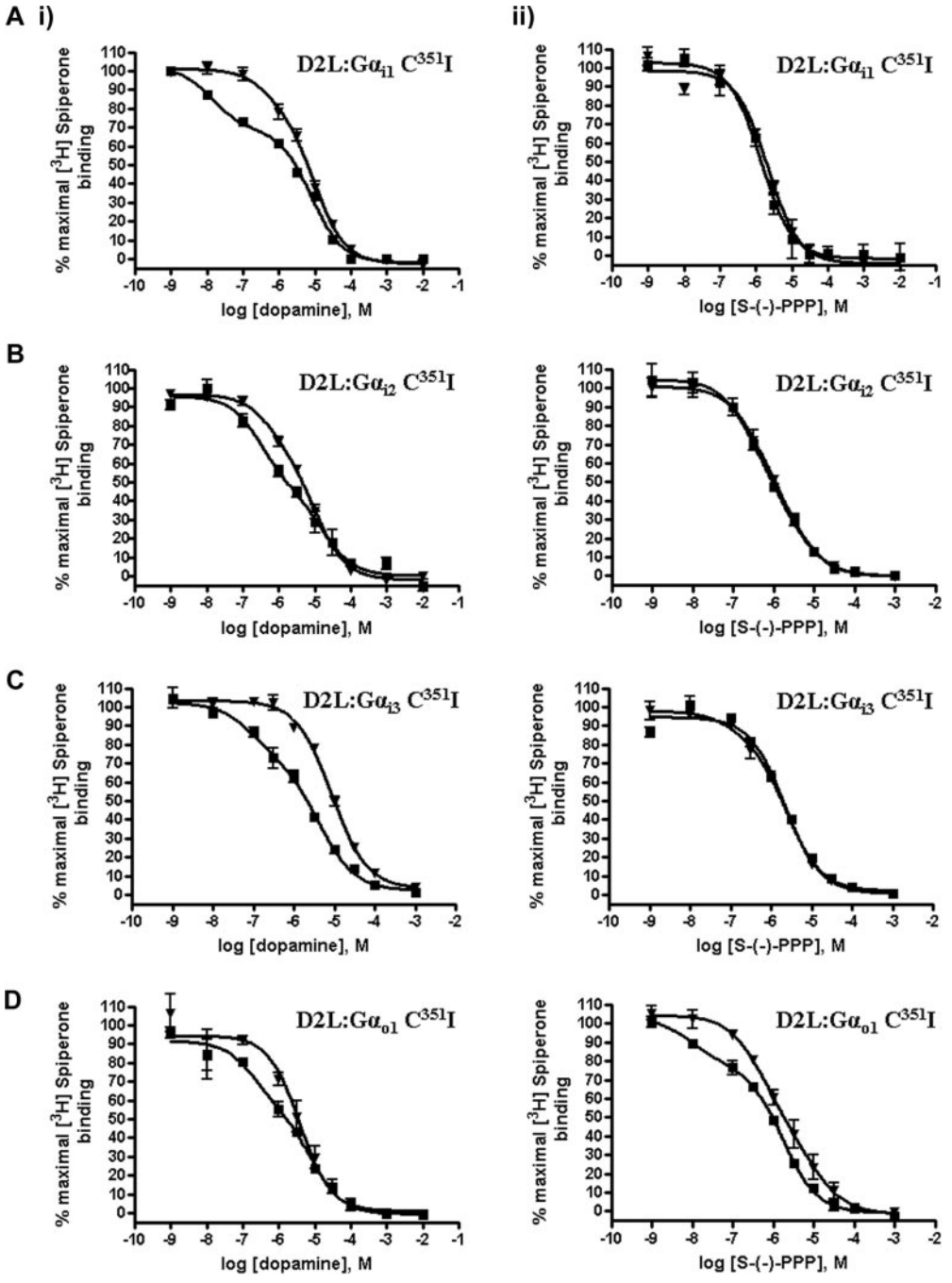

Fig. 2. In ligand binding studies, *S*-(−)-3PPP displays both low- and high-affinity states only at the D2L-Gα_{o1} fusion protein. Membranes of pertussis toxin-treated HEK293 cells expressing D2L-Gα_{i1} (A), D2L-Gα_{i2} (B), D2L-Gα_{i3} (C), and D2L-Gα_{o1} (D) were used in competition binding studies using 0.1 nM [^{3}H]spiperone and varying concentrations of either dopamine (i) or *S*-(−)-3PPP (ii). The assays were performed in the absence (■) or presence (▼) of 100 μM GTP. In the absence of GTP, both high- and low-affinity states for dopamine were observed for each fusion protein, whereas this was only true for *S*-(−)-3PPP at the D2L-Gα_{o1} fusion protein. The presence of GTP converted each competition curve to a single monophasic state displaying low affinity for either dopamine or *S*-(−)-3PPP.

tions of *S*-(−)-3-PPP inhibited the capacity of an EC_{50} concentration of dopamine to enhance binding of [^{35}S]GTPγS to both fusion constructs with pIC_{50} = 4.68 ± 0.07 (D2L-Cys^{352}Ile Gα_{i2}) and 5.09 ± 0.12 (D2L-Cys^{351}Ile Gα_{o1}) (Fig. 3A). However, the maximal effect of *S*-(−)-3-PPP at D2L-Cys^{351}Ile Gα_{o1} in this assay confirmed its partial agonist action at Gα_{o1} because it failed to reduce binding of [^{35}S]GTPγS to the level observed in the absence of dopamine. By contrast, at D2L-Cys^{352}Ile Gα_{i2}, *S*-(−)-3-PPP completely blocked dopamine stimulation of [^{35}S]GTPγS and, indeed, acted as an efficacious inverse agonist (Fig. 3A). Spiperone is frequently described as an inverse agonist at the dopamine D_2 receptor, and accordingly, spiperone also acted as an effective inverse agonist at D2L-Cys^{352}Ile Gα_{i2} (Fig. 3B). Furthermore, this ligand also completely reversed the effect of dopamine at D2L-Cys^{351}Ile Gα_{o1} (Fig. 3B). Further studies demonstrated that both spiperone and *S*-(−)-3-PPP also acted as antagonists/inverse agonists at D2L-Cys^{351}Ile Gα_{i1} and D2L-Cys^{351}Ile Gα_{i3} (Fig. 3, C and D).

Cell Lines that Express Gα_{i1}, Gα_{i2}, Gα_{i3}, or Gα_{o1} in an Inducible Manner. Although the fusion proteins described above have a major advantage in defining and ensuring the receptor-to-G protein ratio for each G protein, they are an inherently artificial system. To examine whether *S*-(−)-3-PPP would also behave as a protean agonist at the D2L receptor when regulating different G proteins in a system expressing separated receptor and G protein, we generated a series of HEK293 cell lines based on the Flp-In T-REx system (Ellis et al., 2006; Milasta et al., 2006). In these cell lines, the D2L receptor was expressed stably and constitutively, whereas the pertussis toxin-resistant cysteine to isoleucine forms of Gα_{i1}, Gα_{i2}, Gα_{i3}, and Gα_{o1} were cloned into the Flp-In locus, allowing their expression in an entirely inducible manner from the same single defined chromosomal locus by the addition of tetracycline. In concert with pertussis toxin treatment, to cause ADP-ribosylation of endogenously expressed forms of Gα_i, we anticipated that this would produce a second alternative system in which D2L receptor-mediated stimulation of [^{35}S]GTPγS binding must reflect only activation of a single defined G protein. Initial studies confirmed expression of the G protein of interest in all cases in a "tetracycline-on" fashion (Fig. 4). At a 24-h time point, maximal expression of each G protein was achieved by treatment of the cells with between 0.5 and 1.0 μg/ml tetracycline (Fig. 4). Levels of the D2L receptor constitutively expressed by each of the cell lines were not affected ($p > 0.05$) by tetracy-

TABLE 3

The binding characteristics of dopamine and *S*-(−)-3-PPP with D2L receptor-G protein fusions

Membranes from HEK293 cells transfected to express each of the D2L-G protein fusions transiently were used in competition binding studies using [^{3}H]spiperone and varying concentrations of either dopamine or *S*-(−)-3PPP. Data were analyzed as described under *Materials and Methods*. p*K* values are provided where appropriate for both high (h) and low (l) affinity sites or where the data did not warrant a "two-site" fit simply as pK_l. In the cases in which a two-site fit provided a significant improvement in data fit, the proportion of high-affinity sites (% R_h) is provided. In all cases, a one site fit was appropriate for data obtained in the presence of GTP (pK_l GTP). Data represent means (±) S.E.M. from a minimum of three independent experiments.

Receptor:G Protein Fusion	D2L Gα_{i1}		D2L Gα_{i2}		D2L Gα_{i3}		D2L Gα_{o1}	
	Dopamine	*S*-(−)-3-PPP	Dopamine	*S*-(−)-3-PPP	Dopamine	*S*-(−)-3-PPP	Dopamine	*S*-(−)-3-PPP
pK_h (±S.E.M.)	7.12 (0.11)		7.20 (0.12)		7.52 (0.13)		7.67 (0.35)	8.44 (0.15)
pK_l (±S.E.M.)	5.75 (0.08)	6.27 (0.06)	5.63 (0.17)	6.47 (0.11)	5.58 (0.12)	6.26 (0.07)	5.82 (0.18)	6.24 (0.07)
%R_h	44 (7)		55 (4)		36 (4)		29 (4)	29 (2)
pK_{iGTP} (±S.E.M.)	5.53 (0.01)	6.23 (0.02)	5.81 (0.01)	6.52 (0.11)	5.50 (0.11)	6.27 (0.11)	5.86 (0.07)	6.3 (0.01)

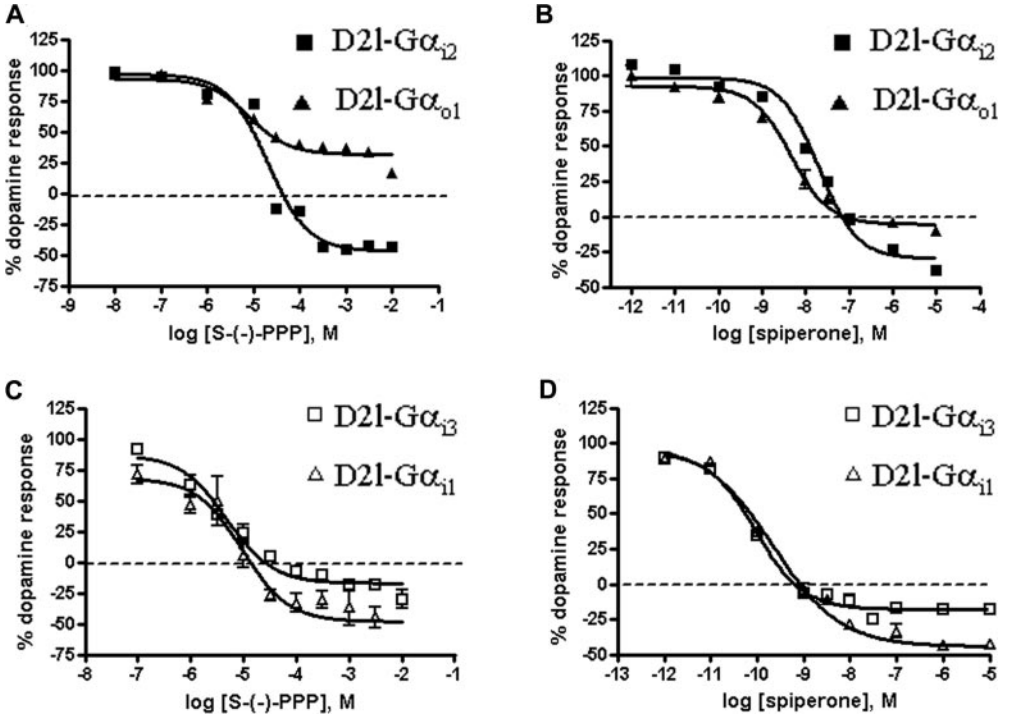

Fig. 3. *S*-(−)-3PPP is an agonist at D2L-Gα_{o1} but an antagonist/inverse agonist for the other D2L-G protein fusion proteins. A and B, membranes from pertussis toxin-treated HEK293 cells expressing either D2L-Gα_{o1} (▲) or D2L-Gα_{i2} (■) were used in [^{35}S]GTPγS binding studies. Dopamine at an EC_{50} concentration for each fusion (0.3 μM D2L-Gα_{o1}, 3 μM D2L-Gα_{i2}) along with varying concentration of either *S*-(−)-3PPP (A) or spiperone (B) were used. On the *y*-axis, 100 is the stimulation produced by dopamine in the absence of a second ligand, and 0 the basal activity in the absence of ligands. C and D, experiments equivalent to those of A and B were performed with membranes expressing D2L-Gα_{i1} (△) or D2L-Gα_{i3} (□). C, studies with *S*-(−)-3PPP. D, studies with spiperone.

cline-induced turn-on of the G proteins (Table 4). As anticipated, in membranes of pertussis toxin-treated cells that were not treated with tetracycline, there was no capacity of dopamine to stimulate binding of [^{35}S]GTPγS, whereas in equivalent membranes of cells treated with tetracycline to cause the expression of Cys^{352}IleGα_{i2} dopamine produced a robust stimulation of [^{35}S]GTPγS binding (Fig. 5A). Although this effect of dopamine was both substantial and concentration-dependent (Fig. 5B), *S*-(−)-3-PPP was unable to enhance [^{35}S]GTPγS binding at all and, indeed, tended to reduce basal [^{35}S]GTPγS binding (Fig. 5B). In membranes of pertussis toxin-treated cells expressing the D2L receptor and induced to express Cys^{351}IleGα_{o1}, dopamine was also able to stimulate binding of [^{35}S]GTPγS (Fig. 6A) in a concentration-dependent fashion (Fig. 6B), and now *S*-(−)-3-PPP also enhanced [^{35}S]GTPγS binding but functioned as a partial agonist (Fig. 6B).

The ability of dopamine to compete with [^{3}H]spiperone to bind the D2L receptor was monophasic, of low-affinity, and insensitive to GTP in membranes of pertussis toxin-treated cells not induced to express Cys^{352}Ile Gα_{i2} (Fig. 7A). However, tetracycline induction of Cys^{352}Ile Gα_{i2} expression resulted in the appearance of a high-affinity site for dopamine that was eliminated in the presence of GTP (Fig. 7A). By contrast, both with and without tetracycline induction of Cys^{352}Ile Gα_{i2} expression, the capacity of *S*-(−)-3-PPP to compete with [^{3}H]spiperone was monophasic and unaffected by the presence of GTP (Fig. 7B). In equivalent membranes of pertussis toxin-treated cells that allowed inducible expression of Cys^{351}IleGα_{o1}, dopamine again identified both high- and low-affinity sites in [^{3}H]spiperone competition binding

TABLE 4

Expression levels (±S.E.M.) of the D2L receptor are unaffected by expression of various G proteins

Flp-In-T-REx HEK293 cells were established able to express pertussis toxin-resistant forms of each of Gα_{i1}, Gα_{i2}, Gα_{i3}, and Gα_{o1} in a tetracycline-dependent fashion (see Fig. 4 for details). These cells were further transfected to express the D2L receptor stably and constitutively. Individual clones were subsequently isolated. After pertussis toxin treatment and treatment with or without 1 μg/ml tetracycline, saturation [^{3}H]spiperone ligand binding studies were performed on membrane preparation as detailed under *Materials and Methods*. Data represent means ± S.E.M. of both B_{max} and K_d values from at least three experiments performed on different membrane preparations. Induction of G protein expression did not significantly alter D2L receptor expression levels.

Cell Line	[^{3}H]Spiperone Binding			
	− 1 μg/ml Tetracycline		+ 1 μg/ml Tetracycline	
	B_{max}	K_d	B_{max}	K_d
	fmol/mg	*nM*	*fmol/mg*	*nM*
D2L + Gα_{i1}	988 (108)	0.02 (0.01)	1156 (261)	0.03 (0.01)
D2L + Gα_{i2}	1448 (191)	0.02 (0.01)	1861 (225)	0.04 (0.01)
D2L + Gα_{i3}	538 (52)	0.03 (0.01)	510 (120)	0.02 (0.02)
D2L + Gα_{o1}	3790 (396)	0.06 (0.02)	3864 (249)	0.07 (0.02)

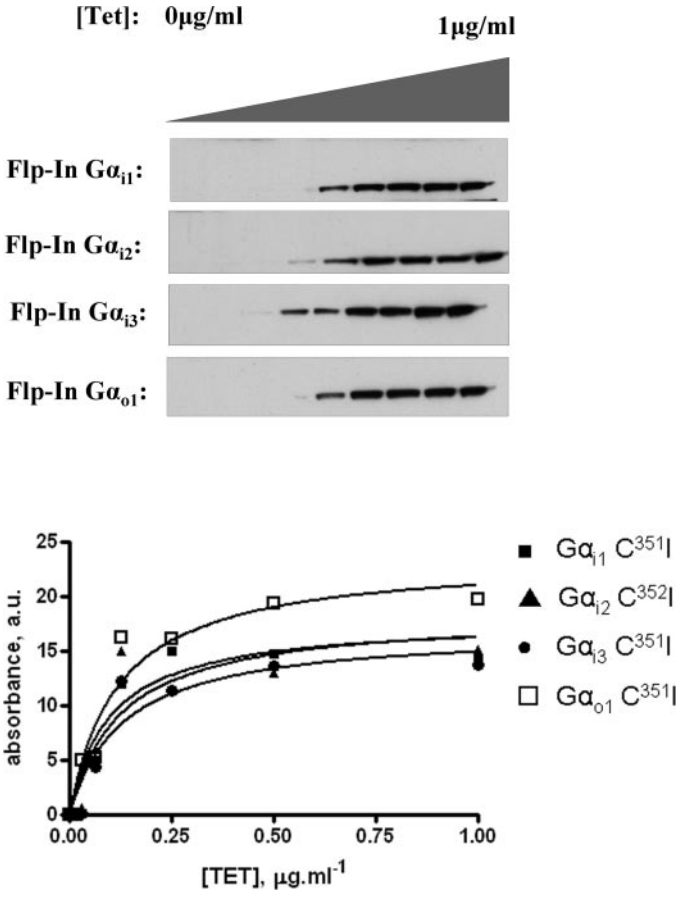

Fig. 4. Characterization of Flp-In T-REx cells harboring pertussis toxin-insensitive mutant G proteins at the Flp-In locus. Flp-In T-REx cell lines were established with either Cys^{351}Ile Gα_{i1}, Cys^{352}Ile Gα_{i2}, Cys^{351}Ile Gα_{i3}, or Cys^{351}Ile Gα_{o1} cloned into the Flp-In locus. These cells were further transfected to constitutively and stably express the D2L receptor (see Table 4 for details). These cells were treated with concentrations of tetracycline (TET) between 0 and 1.0 μg/ml for 24 h. Cell membranes were then prepared, resolved by SDS-polyacrylamide gel electrophoresis, and immunoblotted to detect each individual G protein (top). Densitometric scans were used to quantitate relative expression levels of the G proteins (bottom).

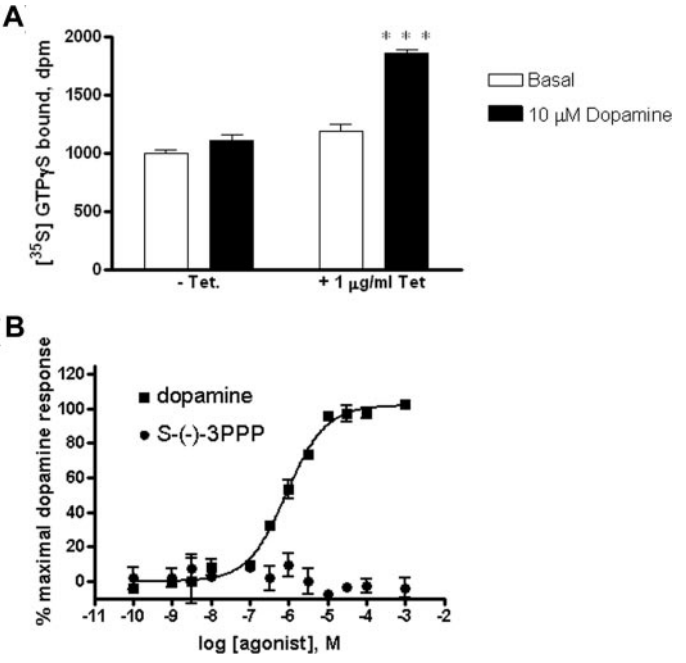

Fig. 5. D2L receptor stimulates [^{35}S]GTPγS binding to Cys^{352}Ile Gα_{i2} in membranes of pertussis toxin-treated Flp-In T-REx cells only when G protein expression is induced. Flp-In T-REx HEK293 cells stably expressing the D2L receptor and harboring Cys^{352}Ile Gα_{i2} at the Flp-In locus were treated with or without 1 μg/ml tetracycline for 24 h. Both sets of cells were also treated with pertussis toxin. Membranes from these cells were used to measure basal [^{35}S]GTPγS binding and the effect of 10 μM dopamine on this (A), and to measure the ability of various concentrations of dopamine (■) or *S*-(−)-3PPP (●) to modulate [^{35}S]GTPγS binding after induction of Cys^{352}Ile Gα_{i2} (B). ***, dopamine enhanced binding of [^{35}S]GTPγS, $p < 0.001$.

studies only after treatment with tetracycline (Fig. 8A). As anticipated, the high-affinity site was absent in the presence of GTP (Fig. 8A). It is interesting that although not as pronounced as with dopamine, *S*-(−)-3-PPP also identified both high- and low-affinity states in membranes of cells induced to express $Cys^{351}IleG\alpha_{o1}$ (Fig. 8B).

Finally, we explored the pharmacology of both spiperone and *S*-(−)-3-PPP in membranes of cells expressing the D2L receptor and induced to express either $Cys^{352}Ile\ G\alpha_{i2}$ (Fig. 9A) or $Cys^{351}IleG\alpha_{o1}$ (Fig. 9B). Dopamine-mediated stimulation of [^{35}S]GTPγS binding was completely reversed by both spiperone and *S*-(−)-3-PPP when $Cys^{352}Ile\ G\alpha_{i2}$ was the target (Fig. 9A), but whereas spiperone also fully reversed dopamine stimulation of [^{35}S]GTPγS binding to $Cys^{351}IleG\alpha_{o1}$ (Fig. 9A), *S*-(−)-3-PPP produced only partial inhibition (Fig. 9B).

Discussion

A number of recent studies have provided evidence that different agonist ligands at a single GPCR can selectively identify and stabilize distinct confirmations or sets of conformations of the receptor (Ghanouni et al., 2001; Krueger et al., 2005; Yao et al., 2006). This can result in differences in the ability of individual agonists to control pairs of signal transduction pathways that are modulated by that GPCR (Perez and Karnik, 2005). Such observations are important in that they provide suggestions of mechanisms that may explain differential functional properties of individual agonist ligands that are believed to be selective for a single GPCR and give insights into the flexibility of GPCR structures. Furthermore, a number of studies have identified differences in the potency or efficacy of agonist ligands to stimulate different G proteins if a GPCR has the potential to interact with more than one G protein (Cussac et al., 2002). Examples of this have been reported for GPCRs that interact selectively with members of the G_i group of pertussis toxin-sensitive G proteins, including the dopamine D_2 receptor (Cordeaux et al., 2001). Because the members of this G protein subfamily that are widely expressed are similar in sequence and, hence, presumably in structure, it is not surprising that many GPCRs can interact with and activate more than one member of the family.

Studies of potential selectivity are hampered by the coexpression of a number of these G proteins in essentially all mammalian cells and tissues. One means to overcome this has been to use insect cell systems (Clawges et al., 1997; Cordeaux et al., 2001) in which levels of expression, or the sequence conservation, of such G proteins is low. When mam-

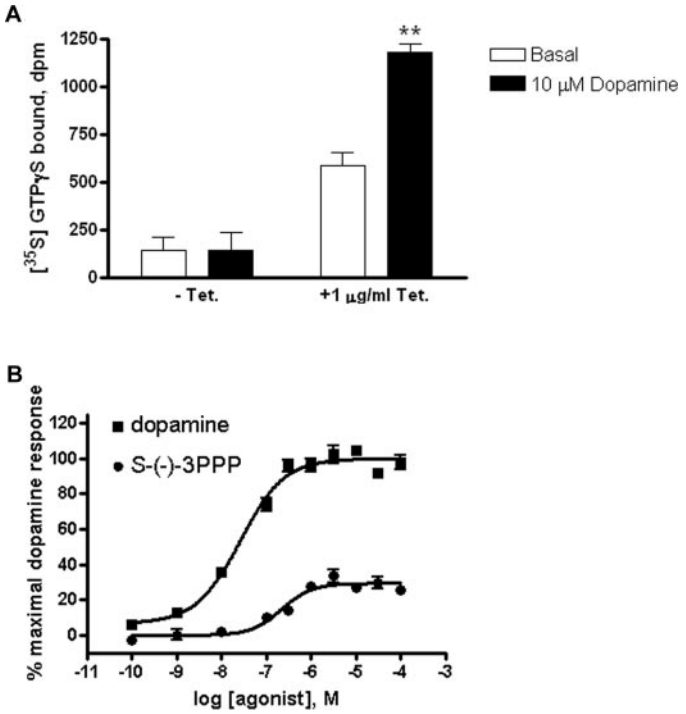

Fig. 6. D2L receptor stimulates [^{35}S]GTPγS binding to $Cys^{351}Ile\ G\alpha_{o1}$ in membranes of pertussis toxin-treated Flp-In T-REx cells only when G protein expression is induced. Flp-In T-REx HEK293 cells stably expressing the D2L receptor and harboring $Cys^{351}Ile\ G\alpha_{o1}$ at the Flp-In locus were treated with or without 1 μg/ml tetracycline for 24 h. Both sets of cells were also treated with pertussis toxin. Membranes from these cells were used to measure basal [^{35}S]GTPγS binding and the effect of 10 μM dopamine on this (A) and to measure the ability of various concentrations of dopamine (■) or *S*-(−)-3PPP (●) to modulate [^{35}S]GTPγS binding after induction of $Cys^{351}Ile\ G\alpha_{o1}$ (B). **, dopamine enhanced binding of [^{35}S]GTPγS, $p < 0.01$.

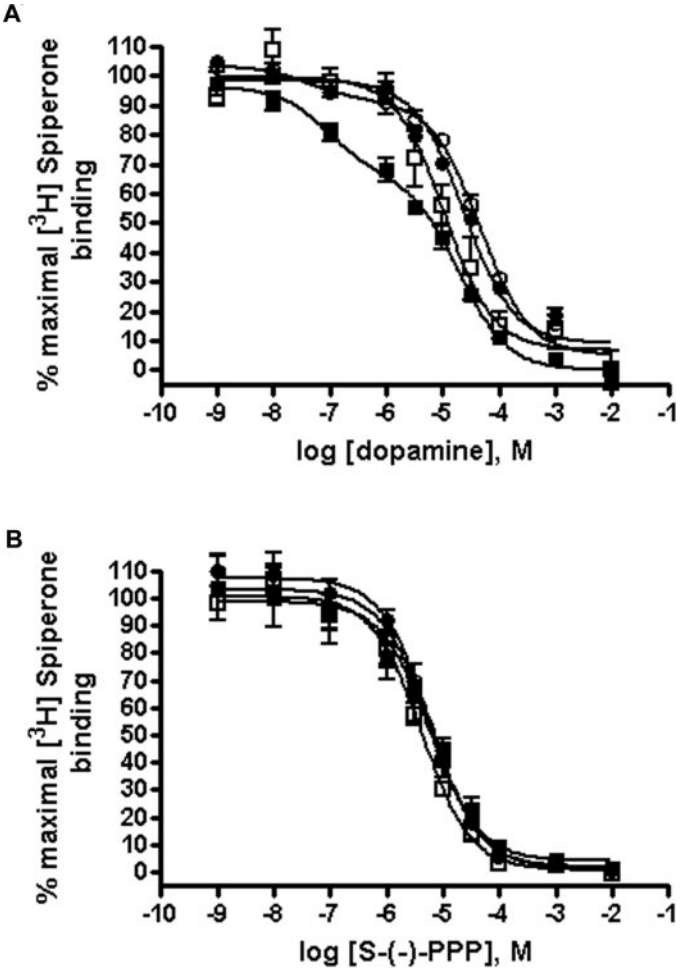

Fig. 7. A high-affinity binding site for dopamine but not for *S*-(−)-3PPP appears with induced expression of $Cys^{352}Ile\ G\alpha_{i2}$. Flp-In T-REx HEK293 cells stably expressing the D2L receptor and harboring $Cys^{352}Ile\ G\alpha_{i2}$ at the Flp-In locus were treated with (filled symbols) or without (open symbols) tetracycline and pertussis toxin as in Fig. 5. Membranes from these cells were employed in competition binding assays using 0.1 nM [^{3}H]spiperone and varying concentrations on either dopamine (A) or *S*-(−)-3PPP (B) in the absence (squares) or presence (circles) of 100 μM GTP.

malian G proteins are introduced into such cells along with a GPCR of interest, activation is largely restricted to the exogenous G protein. An alternative that allows the use of mammalian cell lines has been to use pertussis toxin-resistant variants (Ghahremani et al., 1999; Wise et al., 1999) in which the cysteine residue that is the target for toxin-mediated ADP-ribosylation is altered to another amino acid but retains the capacity to interact with GPCRs. When such mutants are expressed in mammalian cells, treatment of the cells with pertussis toxin results in ADP-ribosylation of the endogenously expressed forms of G_i and an inability of GPCRs to cause their activation. Studies detailed previously on $G\alpha_{i1}$ replaced the relevant cysteine with every other natural amino acid and assessed the impact on GPCR-mediated activation (Bahia et al., 1998), and similar studies were subsequently performed with $G\alpha_{i3}$ (Dupuis et al., 2001).

However, these widely used approaches are still not ideal for detailed studies on differential agonist actions at different G proteins if expression levels are not carefully controlled. Most importantly, although it is well-established that alterations in GPCR-to-G protein ratios can alter ligand function and receptor pharmacology (Milligan, 2000), this can be a challenge to control. For example, in previous studies examining interactions between the D2L and different G protein α subunits in insect Sf9 cells, receptor-to-G protein ratios ranging from 1:3 to 1:14 were reported for the different G proteins (Gazi et al., 2003). In the current studies, we therefore combined the use of pertussis toxin-resistant forms of the various G_i-like G proteins with both GPCR-G protein fusion technologies (Milligan et al., 2004) and with the inducible expression of individual G proteins from a single defined site of chromosomal integration in Flp-In T-REx HEK293 cells (Ellis et al., 2006; Milasta et al., 2006).

Although an artificial system, GPCR-G protein fusions can greatly improve signal-to-background in [^{35}S]GTPγS binding studies (Milligan, 2003; Milligan et al., 2006), and they ensure the same receptor to G protein stoichiometry for each construct. Although the Flp-In T-REx HEK293 cells cannot ensure exactly the same level of expression of each G protein, each G protein is produced from the same single chromosomal location, and this overcomes the potential for different clones to have more than a single site of integration of the cDNA of interest and that different sites of integration might alter the effectiveness of expression. Furthermore, the inducible nature of expression from this locus, combined with the use of pertussis toxin-insensitive mutants and pertussis

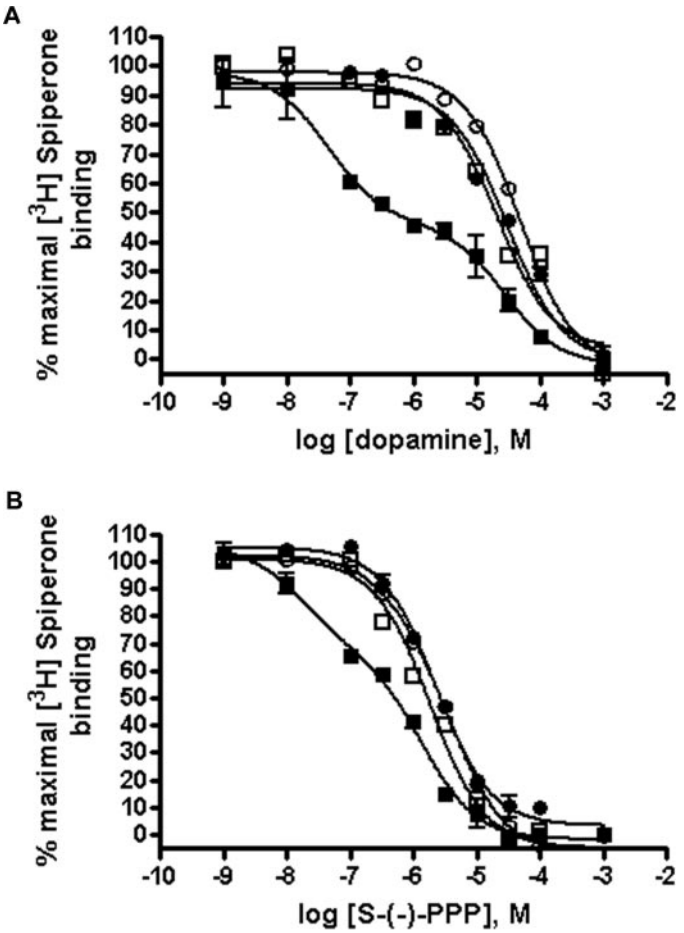

Fig. 8. A high affinity binding site for both dopamine and for *S*-(−)-3PPP appears with induced expression of Cys^{351}Ile Gαo1. Flp-In T-REx HEK293 cells stably expressing the D2L receptor and harboring Cys^{351}Ile $G\alpha_{o1}$ at the Flp-In locus were treated with (filled symbols) or without (open symbols) tetracycline and pertussis toxin as in Fig. 5. Membranes from these cells were used in competition binding assays using 0.1 nM [^{3}H]spiperone and varying concentrations on either dopamine (A) or *S*-(−)-3PPP (B) in the absence (squares) or presence (circles) of 100 μM GTP.

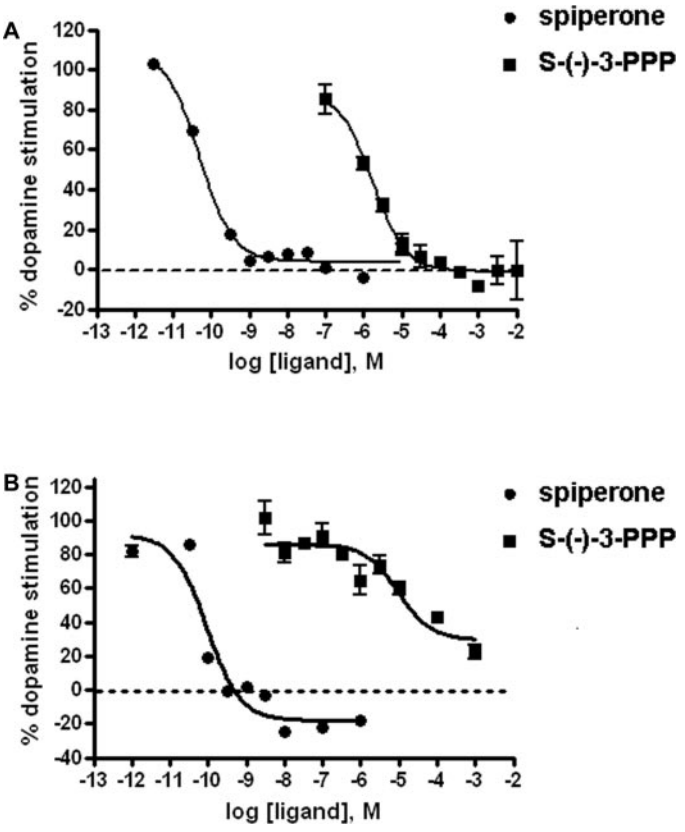

Fig. 9. *S*-(−)-3PPP is an antagonist of D2L-mediated activation of Cys^{352}Ile $G\alpha_{i2}$ but a partial agonist for Cys^{351}Ile $G\alpha_{o1}$. Flp-In T-REx HEK293 cells stably expressing the D2L receptor and harboring Cys^{352}Ile $G\alpha_{i2}$ (A) or Cys^{351}Ile $G\alpha_{o1}$ (B) at the Flp-In locus were treated with tetracycline and pertussis toxin as in Fig. 5. Dopamine (0.1 μM for Cys^{351}Ile $G\alpha_{o1}$, 1 μM for Cys^{352}Ile $G\alpha_{i2}$) was used to stimulate [^{35}S]GTPγS binding, and the effects of varying concentrations of spiperone (●) or *S*-(−)-3PPP (■) on this were assessed. As in Fig. 3, on the *y*-axis, 100 is the stimulation produced by dopamine in the absence of a second ligand, and 0 the basal activity in the absence of ligands.

toxin pretreatment, provided a null background for expression of each G protein.

As anticipated, the majority of dopamine D_2 receptor agonists stimulated [^{35}S]GTPγS binding to all of the four G proteins, although, as reported by others (Gazi et al., 2003), significant variation in potency and efficacy could be observed. However, although both *p*-tyramine and *S*-(−)-3PPP were agonists, at Cys^{351}IleGα_{o1}, they both failed to act in this manner for the other three Gα subunits. The potency of *p*-tyramine was sufficiently low to limit its usefulness for detailed studies. The higher potency of *S*-(−)-3PPP, however, allowed concentration-response curves to demonstrate that it was able to fully inhibit dopamine-stimulated binding of [^{35}S]GTPγS to Cys^{352}Ile Gα_{i2} and, indeed, acted as an inverse agonist. By contrast, even at maximally effective concentrations, *S*-(−)-3PPP was unable to fully reverse dopamine-stimulated binding of [^{35}S]GTPγS to Cys^{351}Ile Gα_{o1}, consistent with the direct measures of its partial agonist activity. In competition studies using two agonist ligands of varying efficacy, full receptor occupancy by the ligand with lower efficacy is expected to result in a direct measure of the efficacy of that ligand.

In further support of the protean effect of *S*-(−)-3PPP at different G proteins, ligand binding studies identified both high- and low-affinity states of the D2L receptor for dopamine with each of the four G proteins but distinct high- and low-affinity states of the receptor for *S*-(−)-3PPP only for Cys^{351}Ile Gα_{o1}. Similar results were obtained using both the receptor-G protein fusions and cells able to produce the G protein of choice on demand. Indeed, the Flp-In T-REx cells were particularly useful in this regard because, with pertussis toxin treatment but without tetracycline-induction of expression of an appropriate G protein, all of the [^{3}H]spiperone binding sites displayed monophasic and low-affinity interactions with both dopamine and *S*-(−)-3PPP, whereas induction of expression resulted in the development of a high-affinity state for dopamine, no matter which of the G proteins was expressed. By contrast, only expression of Cys^{351}Ile Gα_{o1} resulted in the appearance of a high-affinity state for *S*-(−)-3PPP.

In general, the potency of the ligands used was higher for activation of Gα_o than for Gα_{i2}. There were not statistically valid differences between the values obtained for Gα_{i1}, Gα_{i2}, and Gα_{i3} for enough compounds to allow us to convincingly state rank-order potency differences (which might reflect selective stabilization of distinct states of the receptor) for interactions with Gα_{i1} versus Gα_{i2}, for example. This may reflect that the amino acid sequence identities for Gα_{i1}, Gα_{i2}, and Gα_{i3} are all between 86 and 94%, whereas for each of these against Gα_o, sequence identity lies between 70 and 73%. It might, therefore, be postulated that greater variation in ligand conformational states could be observed for interactions with Gα_o versus the others rather than between Gα_{i1}, Gα_{i2}, and Gα_{i3}.

These studies may have implications for the action of *S*-(−)-3PPP. This ligand has been described to have agonist and antagonist properties in physiologically relevant endpoints (Arnt et al., 1983; Hjorth et al., 1983). Of course, one explanation of such observations might relate to its partial agonist function at Gα_{o1} and antagonist/inverse agonist function at Gα_{i1}, Gα_{i2}, and Gα_{i3}. In two studies with *S*-(−)-3-PPP, patients with schizophrenia showed improvements in both positive and negative symptoms but a limited duration of effectiveness (Lahti et al., 1998). It has been postulated that this is due to the action of *S*-(−)-3-PPP as a D_2-like dopamine receptor partial agonist and that this would have a "dopamine system stabilization effect," that is, normalization of both dopamine hypo- and hyperactivity in the pathologically affected dopaminergic tracts observed in patients with schizophrenia (Lieberman, 2004). Likewise, the atypical antipsychotic aripiprazole, which now has Food and Drug Administration approval for the treatment of schizophrenia, has also been characterized as a partial agonist at D_2 receptors and again to have a dopamine stabilization effect (Cosi et al., 2006). However, the intrinsic activity and potency of aripiprazole at the D_2 receptor is both cell line- and assay-dependent. For example aripiprazole has been shown to be a partial agonist for inhibition of cAMP accumulation in a Chinese hamster ovary cell line but an antagonist for [^{35}S]GTPγS binding. Most significant, however, is the observation that, like *S*-(−)-3-PPP, this drug is reported to antagonize postsynaptic D_2 receptors but partially activate presynaptic autoreceptors (Kikuchi et al., 1995). In agreement with these findings, a recent study has demonstrated the differential signaling of aripiprazole for several D2L-mediated pathways (Urban et al., 2007). These observations are consistent with aripiprazole, like *S*-(−)-3-PPP, having differential pharmacology at different signaling pathways, and it will be interesting to ascertain whether aripiprazole has similar protean characteristics in terms of G protein coupling. It will now be interesting to explore the more general contribution of protean effects of clinically relevant ligands that seem to target the same receptor.

Acknowledgments

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References

Akam E and Strange PG (2004) Inverse agonist properties of atypical antipsychotic drugs. *Biochem Pharmacol* **67:**2039–2045.

Aoki C, Go CG, Wu K, and Siekevitz P (1992) Light and electron microscopic localization of alpha subunits of GTP-binding proteins, G_o and Gi, in the cerebral cortex and hippocampus of rat brain. *Brain Res* **596:**189–201.

Arnt J, Bogeso KP, Christensen AV, Hyttel J, Larsen JJ, and Svendsen O (1983) Dopamine receptor agonistic and antagonistic effects of 3-PPP enantiomers. *Psychopharmacology (Berl)* **81:** 199–207.

Azzi M, Charest PG, Angers S, Rousseau G, Kohout T, Bouvier M, and Pineyro G (2003) Beta-arrestin-mediated activation of MAPK by inverse agonists reveals distinct active conformations for G protein-coupled receptors. *Proc Natl Acad Sci USA* **100:**11406–11411.

Bahia DS, Wise A, Fanelli F, Lee M, Rees S, and Milligan G (1998) Hydrophobicity of residue^{351} of the G-protein $G_{i1}\alpha$ determines the extent of activation by the α_{2A}-adrenoceptor. *Biochemistry* **37:**11555–11562.

Berg KA, Maayani S, Goldfarb J, Scaramellini C, Leff P, and Clarke WP (1998) Effector pathway-dependent relative efficacy at serotonin type 2A and 2C receptors: evidence for agonist-directed trafficking of receptor stimulus. *Mol Pharmacol* **54:**94–104.

Cavalli A, Druey KM, and Milligan G (2000) The regulator of G protein signaling RGS4 selectively enhances α_{2A}-adrenoreceptor stimulation of the GTPase activity of $G_{o1}\alpha$ and $G_{i2}\alpha$. *J Biol Chem* **275:**23693–23699.

Clawges HM, Depree KM, Parker EM, and Graber SG (1997) Human 5-HT1 receptor subtypes exhibit distinct G protein coupling behaviors in membranes from Sf9 cells. *Biochemistry* **36:**12930–12938.

Cordeaux Y, Nickolls SA, Flood LA, Graber SG, and Strange PG (2001) Agonist regulation of D_2 dopamine receptor/G protein interaction. Evidence for agonist selection of G protein subtype. *J Biol Chem* **276:**28667–28675.

Cosi C, Carilla-Durand E, Assie MB, Ormiere AM, Maraval M, Leduc N, and Newman-Tancredi A (2006) Partial agonist properties of the antipsychotics SSR181507, aripiprazole and bifeprunox at dopamine D2 receptors: G protein activation and prolactin release. *Eur J Pharmacol* **535:**135–144.

Cussac D, Newman-Tancredi A, Duqueyroix D, Pasteau V, and Millan MJ (2002) Differential activation of Gq/11 and Gi_3 proteins at 5-hydroxytryptamine$_{2C}$ receptors revealed by antibody capture assays: influence of receptor reserve and relationship to agonist-directed trafficking. *Mol Pharmacol* **62:**578–589.

Dupuis DS, Wurch T, Tardif S, Colpaert FC, and Pauwels PJ (2001) Modulation of 5-HT(1A) receptor activation by its interaction with wild-type and mutant g(alphai3) proteins. *Neuropharmacology* **40:**36–47.
Ellis J, Pediani JD, Canals M, Milasta S, and Milligan G (2006) Orexin-1 receptor-cannabinoid CB1 receptor hetero-dimerization results in both ligand-dependent and -independent co-ordinated alterations of receptor localization and function. *J Biol Chem* **281:**38812–38824.
Galandrin S and Bouvier M (2006) Distinct signaling profiles of β1 and β2 adrenergic receptor ligands toward adenylyl cyclase and mitogen-activated protein kinase reveals the pluridimensionality of efficacy. *Mol Pharmacol* **70:**1575–1584.
Gazi L, Nickolls SA, and Strange PG (2003) Functional coupling of the human dopamine D2 receptor with G alpha i1, G alpha i2, G alpha i3 and G alpha o G proteins: evidence for agonist regulation of G protein selectivity. *Br J Pharmacol* **138:**775–786.
Gesty-Palmer D, Chen M, Reiter E, Ahn S, Nelson CD, Wang S, Eckhardt AE, Cowan CL, Spurney RF, Luttrell LM, et al. (2006) Distinct β-arrestin- and G protein-dependent pathways for parathyroid hormone receptor-stimulated ERK1/2 activation. *J Biol Chem* **281:**10856–10864.
Ghahremani MH, Cheng P, Lembo PM, and Albert PR (1999) Distinct roles for Gαi2, Gαi3, and G$\beta\gamma$ in modulation of forskolin- or Gs-mediated cAMP accumulation and calcium mobilization by dopamine D2S receptors. *J Biol Chem* **274:**9238–9245.
Ghanouni P, Gryczynski Z, Steenhuis JJ, Lee TW, Farrens DL, Lakowicz JR, and Kobilka BK (2001) Functionally different agonists induce distinct conformations in the G protein coupling domain of the β2 adrenergic receptor. *J Biol Chem* **276:**24433–24436.
Hjorth S, Carlsson A, Clark D, Svensson K, Wikstrom H, Sanchez D, Lindberg P, Hacksell U, Arvidsson L-E, Johansson A, et al. (1983) Central dopamine receptor agonist and antagonist actions of the enantiomers of 3-PPP. *Psychopharmacology (Berl)* **81:**89–99.
Kenakin T (1995) Agonist-receptor efficacy. II. Agonist trafficking of receptor signals. *Trends Pharmacol Sci* **16:**232–238.
Kenakin T (2001) Inverse, protean, and ligand-selective agonism: matters of receptor conformation. *FASEB J* **15:**598–611.
Kikuchi T, Tottori K, Uwahodo Y, Hirose T, Miwa T, Oshiro Y, and Morita S (1995) 7-(4-[4-(2,3-Dichlorophenyl)-1-piperazinyl]butyloxy)-3,4-dihydro-2(1H)-quinolinone (OPC-14597), a new putative antipsychotic drug with both presynaptic dopamine autoreceptor agonistic activity and postsynaptic D2 receptor antagonistic activity. *J Pharmacol Exp Ther* **274:**329–336.
Krueger KM, Witte DG, Ireland-Denny L, Miller TR, Baranowski JL, Buckner S, Milicic I, Esbenshade TA, and Hancock AA (2005) G protein-dependent pharmacology of histamine H3 receptor ligands: evidence for heterogeneous active state receptor conformations. *J Pharmacol Exp Ther* **314:**271–281.
Lahti AC, Weiler MA, Corey PK, Carlsson A, and Tamminga CA (1998) Antipsychotic properties of the partial dopamine agonist (−)-3-(3-hydroxyphenyl)-N-n-propylpiperidine(preclamol) in schizophrenia. *Biol Psychiatry* **48:**3–11.
Leff P (1996) The two-state model of agonist action: challenges to pharmacological receptor theory. *Proc West Pharmacol Soc 3* **9:**67–68.
Leff P, Scaramellini C, Law C, and McKechnie K (1997) A three-state receptor model of agonist action. *Trends Pharmacol Sci* **18:**355–362.
Lieberman JA (2004) Dopamine partial agonists. A new class of antipsychotic. *CNS Drugs* **18:**251–267.
Milasta S, Pediani J, Appelebe S, Trim S, Wyatt M, Cox P, Fidock M, and Milligan G (2006) Interactions between the Mas-related receptors MrgD and MrgE alter signalling and regulation of MrgD. *Mol Pharmacol* **69:**479–491.
Milligan G (2000) Altering the relative stoichiometry of receptors, G-proteins and effectors: effects on agonist function, in *The Pharmacology of Functional, Biochemical, and Recombinant Receptor Systems* (Kenakin T and Angus JA eds) pp 363–389, Springer, New York.
Milligan G (2003) Extending the utility of [^{35}S]GTPγS binding assays. *Trends Pharmacol Sci* **24:**87–90.
Milligan G, Feng G-J, Ward RJ, Sartania N, Ramsay D, McLean AJ, and Carrillo JJ (2004) G protein-coupled receptor fusion proteins in drug discovery. *Curr Pharmaceut Des* **10:**1989–2001.
Milligan G and IJzerman AP (2000) Stochastic multidimensional hypercubes and inverse agonism. *Trends Pharmacol Sci* **21:**362–363.
Milligan G, Stoddart LA, and Brown AJ (2006) G protein-coupled receptors for free fatty acids. *Cell Signal* **18:**1360–1365.
Perez DM and Karnik SS (2005) Multiple signaling states of G-protein-coupled receptors. *Pharmacol Rev* **57:**147–161.
Urban JD, Vargas GA, von Zastrow M, and Mailman RB (2007) Aripiprazole has functionally selective actions at dopamine D2 receptor-mediated signaling pathways. *Neuropsychopharmacology* **32:**67–77.
Vauquelin G and Van Liefde I (2005) G protein-coupled receptors: a count of 1001 conformations. *Fundam Clin Pharmacol* **19:**45–56.
Wei H, Ahn S, Shenoy SK, Karnik SS, Hunyady L, Luttrell LM, and Lefkowitz RJ (2003) Independent β-arrestin 2 and G protein-mediated pathways for angiotensin II activation of extracellular signal-regulated kinases 1 and 2. *Proc Natl Acad Sci USA* **100:**10782–10787.
Wise A and Milligan G (1997) Rescue of functional interactions between the α_{2A}-adrenoreceptor and acylation-resistant forms of $G_{i1}\alpha$ by expressing the proteins from chimeric open reading frames. *J Biol Chem* **272:**24673–24678.
Wise A, Sheehan M, Rees S, Lee M, and Milligan G (1999) Comparative analysis of the efficacy of A_1 adenosine receptor activation of $G_{i/o}\alpha$ G proteins following co-expression of receptor and G protein and expression of A_1 adenosine receptor-$G_{i/o}\alpha$ fusion proteins. *Biochemistry* **38:**2272–2278.
Yao X, Parnot C, Deupi X, Ratnala VR, Swaminath G, Farrens D, and Kobilka B (2006) Coupling ligand structure to specific conformational switches in the beta$_2$-adrenoceptor. *Nat Chem Biol* **2:**417–422.

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