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Title Page

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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Running title page

Running title: Protean agonism at the D2 receptor

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Abbreviations: 7-OH-DPAT, R(+)-7-Hydroxy-DPAT hydrobromide; D2L, long isoform of the human dopamine D_2 receptor; GPCR, G protein-coupled receptor; GTPγS, guanosine 5'-O-(thiotriphosphate); 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;

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ABSTRACT

A range of ligands displayed agonism at the long isoform of the human dopamine D2 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 $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$. In ligand binding assays dopamine identified both high and low affinity states at each of the dopamine D2 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 where the D2 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 D2 receptor-$G\alpha_{o1}$ fusion with the high affinity state being eliminated by guanine nucleotide. Similarly, although dopamine identified guanine nucleotide-sensitive high affinity states of the D2 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 similar profile as a protean ligand as 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 D2 receptor and may explain *in vivo* actions of this ligand.

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INTRODUCTION

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 models of 'three-state' (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 hetero-trimeric G proteins, a number of ligand-induced signals appear 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 ERK1/2 MAP 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 proteins and differences in agonist pharmacology to regulate signals via different G proteins is

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frequently described 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 D2 receptor has been one of the most studied mono-aminergic 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 greatly influenced 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 employ 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 previously employed 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 employing both fusions of the long isoform of the human dopamine D2 (D2L) receptor with pertussis toxin-resistant Cys-Ile 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

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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 D2 receptor can exist in multiple conformational states and indicate that it is possible to selectively control the nature of signals generated by D2 receptor 'agonists'. Because individual pertussis toxin-sensitive G proteins are expressed differentially pre- and post-synaptically (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 G.E. International (Buckinghamshire, U.K.) and [^{35}S]GTPγS (1250 Ci/mmol) was from PerkinElmer Life and Analytical Sciences (Boston, MA). (+)-butaclamol, dopamine, (-)-quinpirole, *m*-tyramine, *p*-tyramine, *S*-(-)-3-(3-hydroxyphenyl)-*N*-propylpiperidine (S-(-)-PPP), *R*-(+)-3-PPP, *R*-(-)-10,11-dihydroxy-*N*-*n*-propylnorapomorphine (NPA), R(+)-7-Hydroxy-DPAT hydrobromide (7-OH-DPAT) and guanosine 5'-O-(thiotriphosphate)(GTPγS) were purchased from Sigma (Gillingham, Dorset, U.K.). Spiperone hydrochloride was from Tocris (Bristol, U.K.). Oligonucleotides were from ThermoElectron (Ulm, Germany) and all materials for tissue culture were from Invitrogen (Paisley, UK). All other reagents were obtained as indicated.

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D_2 dopamine receptor subcloning into pcDNA3 - The long isoform of the human D_2 dopamine receptor (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 EcoR I (sense) and Xho I (antisense). The resulting PCR fragment was digested with EcoR I and Xho I 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' Kpn I site and 3' EcoR1 site, with a Nco I site between receptor and G-protein α subunit cDNAs. To create D2L:G protein α subunit proteins, the first step was to remove the Nco I 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 CAT GGT CTC CAC AG -3'; antisense, 5'-CT GTG GAG ACC ATG ATG GGA CGG GTC GG -3'. Bold letters indicate altered bases. The PCR product was then digested with Dpn I and transformed into bacteria. In a similar manner Nco I 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

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ATG GGG AGA TTG AAA ATC G -3'; antisense, 5'-C GAT TTT CAA TCT CCC CAT CGC TCT AAT GAT GGC AA -3', $G\alpha_{o1}$ 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 (Nco I -) 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 GGAGTGGAGGATCTTCAGGAAGGC -3'. Underlined bases indicate introduced restriction sites (sense: Kpn I, antisense; Nco I), bases in bold indicate introduced N-terminal Myc tag. The PCR fragment was digested using Kpn I and Nco I.

The α_{2A}-adrenoceptor–Gα fusion proteins (Nco I-) were excised from pcDNA3 using Kpn I and EcoR1, digested with Nco I, and the Gα subunit cDNA 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 Ile (Cys^{351} for $G\alpha_{i2}$) by site-directed mutagenesis. These were cloned into pcDNA3. cDNAs were excised using Kpn I and Apa I ($G\alpha_{i1-3}$) or Apa I ($G\alpha_{o1}$) and subcloned into the pcDNA5/FRT/TO vector (Invitrogen)

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Cell Culture and Transfection—HEK293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 0.292 g/litre L-glutamine and 10% (v/v) newborn calf serum at 37 °C in a 5% CO_2 humidified atmosphere. Cells were grown to 60–80% 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 (Milasta et al., 2006). Clones were screened for G protein expression by Western blotting. To constitutively stably co-express 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 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 1h and resuspended in 15 ml of buffer (10mM Tris, 0.1mM EDTA, pH7.4). Cell suspensions were then homogenized using an Ultra Turrax for 3 x 20 s. The homogenate was centrifuged at 1700×*g* for 10 min and the

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supernatant 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 1ml.

Saturation binding assays using [^{3}H]-spiperone- Cell membranes (10 μg protein) were incubated in triplicate with [^{3}H]spiperone (0.001–2 nM) in a total volume of 1 ml buffer (20 mM HEPES, 6 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, pH 7.4). Non-specific 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 PBS (140 mM NaCl, 10 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4). The filters were soaked in 3 ml scintillation fluid and radioactivity present was determined by liquid scintillation spectrometry.

Agonist competition versus [^{3}H]-spiperone binding - Cell membranes (10 μg protein) were incubated with 0.05 nM [^{3}H]spiperone and various concentrations of agonists, in triplicate, in a final volume of 1 ml buffer (20 mM HEPES, 6 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 40μM ascorbic acid, pH 7.4). Non-specific 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 assessed by the addition of 100 mM NaCl and 100 μM GTP to the buffer.

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[^{35}S]GTPγS binding assays

Cell membranes (10 μg) were incubated in 900 μl buffer (20 mM HEPES, 100 mM NaCl, 6 mM $MgCl_2$, 40μM 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 PBS. 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, 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 (G.E. 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; G.E. Healthcare) at 0.3 nM (total volume of 46 μl), and binding was allowed to proceed at room temperature for four hours. Bound [^{35}S]GTPγS was determined by scintillation counting on a ViewLux ultraHTS Microplate Imager (PerkinElmer).

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Data analysis-Data were analysed using PRISM (GraphPAD Software Inc., San Diego, CA)

RESULTS

Studies with dopamine D_2 receptor-G protein fusions

It has previously been established 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, Cys to Ile, 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 (**Figure 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. Following 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 employing [^{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

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cells mock transfected or transfected to express D2L-Cys^{351}Ile Gα_{o1} and treated or not with pertussis toxin were employed in [^{35}S]GTPγS binding studies. In the absence of D2L-Cys^{351}Ile Gα_{o1}, binding of the nucleotide was low, essentially unaffected by pertussis toxin treatment and not modulated by addition of dopamine (**Figure 1B**). With expression of D2L-Cys^{351}Ile Gα_{o1}, [^{35}S]GTPγS binding in the absence of dopamine was increased, and this level was increased substantially further in the presence of dopamine (**Figure 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 following pertussis toxin treatment the elevation of [^{35}S]GTPγS binding by dopamine remained robust (**Figure 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, N-propylapomorphine (NPA), quinpirole, m-tyramine and R (+) 3-(3-hydroxyphenyl)-N-propylpiperidine (R-(+)-3-PPP) (**Figure 1C)**, although, when compared to 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 Gα_{o1} (**Table 2**) whilst the potency of quinpirole was particularly low at D2L-Cys^{352}Ile Gα_{i2} (**Table 2**). Unlike the ligands mentioned above, although both p-tyramine and S (-) 3-(3-hydroxyphenyl)-N-propylpiperidine (S-(-)-3-PPP) displayed agonism at the D2L-Cys^{351}Ile Gα_{o1} fusion (**Table 2**), they did not enhance

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[^{35}S]GTPγS binding to any of the other fusions. Whilst p-tyramine displayed greater agonist efficacy than S-(-)-3-PPP at the D2L-Cys^{351}Ile Gα_{o1} fusion, subsequent detailed studies employed 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 (**Figure 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 (**Figure 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} where a bi-phasic 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 amongst the 4 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 concentrations 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}) (**Figure 3A**). However, the maximal effect of S-(-)-3-PPP at D2L-Cys^{351}Ile Gα_{o1} in this assay

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confirmed its partial agonist action at $G\alpha_{o1}$, 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 $G\alpha_{i2}$ S-(-)-3-PPP completely blocked dopamine stimulation of [^{35}S]GTPγS and, indeed, acted as an efficacious inverse agonist (**Figure 3A**). Spiperone is frequently described as an inverse agonist at the dopamine D2 receptor and, accordingly, spiperone also acted as an effective inverse agonist at D2L-Cys^{352}Ile $G\alpha_{i2}$ (**Figure 3B**). Furthermore, this ligand also completely reversed the effect of dopamine at D2L-Cys^{351}Ile $G\alpha_{o1}$ (**Figure 3B**). Further studies demonstrated that both spiperone and S-(-)-3-PPP also acted as antagonists/inverse agonists at D2L-Cys^{351}Ile $G\alpha_{i1}$ and D2L-Cys^{351}Ile $G\alpha_{i3}$ (**Figures 3C, 3D**).

Cell lines that express $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$ or $G\alpha_{o1}$ 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 if 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 (Milasta et al., 2006, Ellis et al., 2006). In these cell lines the D2L receptor was expressed stably and constitutively whilst the pertussis toxin-resistant Cys to Ile forms of $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$ and $G\alpha_{o1}$ were cloned into the Flp-In locus, allowing their expression in an entirely inducible manner from the same, single, defined chromosomal locus by addition of tetracycline. In concert with pertussis toxin treatment, to cause ADP-ribosylation of endogenously expressed

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forms of '$G\alpha_i$', we anticipated 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 (**Figure 4**). At a 24 h time point, maximal expression of each G protein was achieved by treatment of the cells with between 0.5-1.0 μg/ml tetracycline (**Figure 4**). Levels of the D2L receptor constitutively expressed by each of the cell lines were not affected ($p > 0.05$) by tetracycline-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, whilst in equivalent membranes of cells treated with tetracycline to cause expression of Cys^{352}Ile$G\alpha_{i2}$ dopamine produced a robust stimulation of [^{35}S]GTPγS binding (**Figure 5A**). Whilst this effect of dopamine was both substantial and concentration-dependent (**Figure 5B**), S-(-)-3-PPP was unable to enhance [^{35}S]GTPγS binding at all, and indeed, tended to reduce basal [^{35}S]GTPγS binding (**Figure 5B**). In membranes of pertussis toxin-treated cells expressing the D2L receptor and induced to express Cys^{351}Ile$G\alpha_{o1}$ dopamine was also able to stimulate binding of [^{35}S]GTPγS (**Figure 6A**) in a concentration-dependent fashion (**Figure 6B**) and now S-(-)-3-PPP also enhanced [^{35}S]GTPγS binding, but functioned as a partial agonist (**Figure 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 $G\alpha_{i2}$ (**Figure 7A**). However, tetracycline induction of Cys^{352}Ile $G\alpha_{i2}$ expression resulted in the

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appearance of a high affinity site for dopamine that was eliminated in the presence of GTP (**Figure 7A**). By contrast, both with and without tetracycline induction of $Cys^{352}Ile\ G\alpha_{i2}$ expression the capacity of S-(-)-3-PPP to compete with [^{3}H]spiperone was monophasic and unaffected by the presence of GTP (**Figure 7B**). In equivalent membranes of pertussis toxin-treated cells that allowed inducible expression of $Cys^{351}IleG\alpha_{o1}$, dopamine again identified both high and low affinity sites in [^{3}H]spiperone competition binding studies only following treatment with tetracycline (**Figure 8A**). As anticipated, the high affinity site was absent in the presence of GTP (**Figure 8A**). Interestingly, 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 $Cys^{351}IleG\alpha_{o1}$ (**Figure 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 $Cys^{352}Ile\ G\alpha_{i2}$ (**Figure 9A**) or $Cys^{351}IleG\alpha_{o1}$ (**Figure 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 (**Figure 9A**) but whilst spiperone also fully reversed dopamine stimulation of [^{35}S]GTPγS binding to $Cys^{351}IleG\alpha_{o1}$ (**Figure 9A**) S-(-)-3-PPP produced only partial inhibition (**Figure 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 D2 receptor (Cordeaux et al., 2001). As 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 co-expression of a number of these G proteins in essentially all mammalian cells and tissues. One means to overcome this has been to employ insect cell systems (Clawges et al., 1997, Cordeaux et al., 2001) where levels of expression, or the sequence conservation, of such G proteins is low. When mammalian 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 use of mammalian cell lines, has been to use pertussis toxin-resistant variants (Wise et al., 1999, Ghahremani et al., 1999) in which the Cys 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. Previous detailed studies on $G\alpha_{i1}$ replaced the relevant Cys 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., 1999).

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

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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 toxin pre-treatment, provided a null background for expression of each G protein.

As anticipated, the majority of dopamine D2 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}IleG$\alpha_{o1,}$ 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 employing 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

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for dopamine with each of the four G proteins but distinct high and low affinity states of the receptor for S-(-)-3PPP only for $Cys^{351}Ile$ $G\alpha_{o1}$. 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, whilst 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 $Cys^{351}Ile$ $G\alpha_{o1}$ 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 $G\alpha_o$ than for $G\alpha_{i2}$. There were not statistically valid differences between the values obtained for $G\alpha_{i1}$, $G\alpha_{i2}$ and $G\alpha_{i3}$ for enough compounds to allow us to convincingly state rank-order potency differences (that might reflect selective stabilization of distinct states of the receptor) for interactions with $G\alpha_{i1}$ versus $G\alpha_{i2}$ for example. This may reflect that the amino acid sequence identities for $G\alpha_{i1}$, $G\alpha_{i2}$ and $G\alpha_{i3}$ are all between 86% and 94%, whereas for each of these against $G\alpha_o$, sequence identity lies between 70-73%. It might, therefore, be postulated that greater variation in ligand conformational states could be observed for interactions with $G\alpha_o$ versus the others rather than between $G\alpha_{i1}$, $G\alpha_{i2}$ and $G\alpha_{i3}$.

These studies may have implications for the action of S-(-)-3PPP. This ligand has been described to have agonist and antagonist properties in physiologically

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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 $G\alpha_{o1}$ and antagonist/inverse agonist function at $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$. 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' i.e. normalization of both dopamine hypo- and hyperactivity in the pathologically affected dopaminergic tracts observed in schizophrenic patients (Lieberman, 2004). Similarly, the atypical antipsychotic aripiprazole which now has FDA 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 CHO cell line but an antagonist for $[^{35}S]GTP\gamma S$ binding. Most significant, however, is the observation that, like S-(-)-3-PPP, this drug is reported to antagonize post-synaptic D_2 receptors but partially activate pre-synaptic auto-receptors (Kikuchi et al., 1995). In agreement with these findings, a recent study has demonstrated the differential signalling of aripiprazole for several D_{2L} mediated pathways (Urban et al., 2007). These observations are consistent with aripiprazole, like S-(-)-3-PPP, having differential pharmacology at different signalling pathways, and it will be interesting to ascertain if aripiprazole has similar 'protean' characteristics in terms of G-protein coupling. It will now be interesting to explore the

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more general contribution of 'protean' effects of clinically relevant ligands that appear to target the same receptor.

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Legends for Figures

Figure 1

G protein fusion proteins identify agonists and protean ligands at the D2L receptor

A. A series of fusion proteins was generated by linking Cys-Ile (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) (25ng/ml, 24 h). Membranes of these cells were employed in [^{35}S]GTPγS binding studies in the absence (open bars) or presence (filled bars) of 100 μM dopamine.

C. Each of the fusion proteins (**i**) D2L-$G\alpha_{i1}$, (**ii**) D2L-$G\alpha_{i2}$, (**iii**) D2L-$G\alpha_{i3}$, (**iv**) D2L-$G\alpha_{o1}$ was expressed transiently in HEK293 cells. Following pertussis toxin treatment (25ng/ml, 24 h) cells were harvested, membranes generated and [^{35}S]GTPγS binding studies 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 (●), 7-OH DPAT (◊)) reported to have affinity and efficacy at dopamine D2 receptors. Data are representative and full details are provided in **Table 2**.

Figure 2

In ligand binding studies S-(-)-3PPP displays both low and high affinity states only at the D2L-$G\alpha_{o1}$ fusion protein

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Membranes of pertussis toxin-treated HEK293 cells expressing (**A**) D2L-Gα_{i1} , (**B**) D2L-Gα_{i2}, (**C**) D2L-Gα_{i3}, (**D**) D2L-Gα_{o1} were used in competition binding studies employing 0.1 nM [^{3}H]spiperone and varying concentrations of either (**i**) dopamine or (**ii**) S-(-)-3PPP. The assays were performed in the absence (**squares**) or presence (**triangles**) of 100μM 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.

Figure 3

S-(-)-3PPP is an agonist at D2L-Gα_{o1} but an antagonist/inverse agonist for the other D2L-G protein fusion proteins

A, **B.** Membranes from pertussis toxin-treated HEK293 cells expressing either D2L-Gα_{o1} (**filled triangles**) or D2L-Gα_{i2} (**filled squares**) 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 employed. 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, D. Equivalent experiments to those of **A** and **B** were performed with membranes expressing D2L-Gα_{i1} (**open triangles**) or D2L-Gα_{i3} (**open squares**). **C**. Studies with S-(-)-3PPP, **D**, studies with spiperone.

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Figure 4

Characterization of Flp-In T-REx cells harbouring 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-PAGE and immunoblotted to detect each individual G protein (**upper panel**). Densitometric scans were used to quantitate relative expression levels of the G proteins (**lower panel**).

Figure 5

D2L receptor stimulates [^{35}S]GTPγS binding to Cys^{352}Ile Gα_{i2} in membranes of pertussis toxin-treated Flp-In T-REx cell only when G protein expression is induced

Flp-In T-REx HEK293 cells stably expressing the D2L receptor and harbouring Cys^{352}Ile Gα_{i2} at the Flp-In locus were treated with or without 1μg/ml tetracycline for 24 h. Both sets of cells were also treated with pertussis toxin. Membranes from these cells were (**A**), used to measure basal [^{35}S]GTPγS binding and the effect of 10 μM dopamine on this, (**B**) to measure the ability of varying concentration of dopamine (**squares**) or S-(-)-3PPP (**circles**) to modulate [^{35}S]GTPγS binding following

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induction of Cys^{352}Ile Gα_{i2}. *** Dopamine enhanced binding of [^{35}S]GTPγS, $p < 0.001$.

Figure 6

D2L receptor stimulates [^{35}S]GTPγS binding to Cys^{351}Ile Gα_{o1} in membranes of pertussis toxin-treated Flp-In T-REx cell only when G protein expression is induced

Flp-In T-REx HEK293 cells stably expressing the D2L receptor and harbouring Cys^{351}Ile Gα_{o1} at the Flp-In locus were treated with or without 1μg/ml tetracycline for 24 h. Both sets of cells were also treated with pertussis toxin. Membranes from these cells were (**A**), used to measure basal [^{35}S]GTPγS binding and the effect of 10 μM dopamine on this, (**B**) to measure the ability of varying concentration of dopamine (**squares**) or S-(-)-3PPP (**circles**) to modulate [^{35}S]GTPγS binding following induction of Cys^{351}Ile Gα_{o1}. ** Dopamine enhanced binding of [^{35}S]GTPγS, $p < 0.01$.

Figure 7

A high affinity binding site for dopamine but not for S-(-)-3PPP appears with induced expression of Cys^{352}Ile Gα_{i2}

Flp-In T-REx HEK293 cells stably expressing the D2L receptor and harbouring Cys^{352}Ile Gα_{i2} at the Flp-In locus were treated with (**filled symbols**) or without (**open symbols**) tetracycline and pertussis toxin as in Figure 5. Membranes from these cells were employed in competition binding assays using 0.1 nM [^{3}H]spiperone and

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varying concentrations on either dopamine (**A**) or S-(-)-3PPP (**B**) in the absence (**squares**) or presence (**circles**) of 100μM GTP.

Figure 8

A high affinity binding site for both dopamine and for S-(-)-3PPP appears with induced expression of $Cys^{351}Ile\ G\alpha_{o1}$

Flp-In T-REx HEK293 cells stably expressing the D2L receptor and harbouring $Cys^{351}Ile\ G\alpha_{o1}$ at the Flp-In locus were treated with (**filled symbols**) or without (**open symbols**) tetracycline and pertussis toxin as in Figure 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 100μM GTP.

Figure 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 harbouring $Cys^{352}Ile\ G\alpha_{i2}$ (**A**) or $Cys^{351}Ile\ G\alpha_{o1}$ (**B**) at the Flp-In locus were treated with tetracycline and pertussis toxin as in Figure 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 (**circles**) or S-(-)-3PPP (**squares**) on this were assessed. As in Figure 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.

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Tables

Table 1

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

Receptor: G Protein fusion	K_d, nM (± S.E.M.)	B_{max} fMol.mg^{-1} (± S.E.M.)
$D_{2l}G\alpha_{i1}$	**0.049** (0.003)	**1500** (49)
$D_{2l}G\alpha_{i2}$	**0.051** (0.003)	**1415** (24)
$D_{2l}G\alpha_{i3}$	**0.057** (0.002)	**1976** (14)
$D_{2l}G\alpha_{o1}$	**0.057** (0.006)	**1508** (43)

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

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Table 2

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

	$D_{2l}G\alpha_{i1}$		$D_{2l}G\alpha_{i2}$		$D_{2l}G\alpha_{i3}$		$D_{2l}G\alpha_{o1}$	
	pEC50, (±s.e.m)	**E_{max}, %DA (±s.e.m)**	**pEC50 (±s.e.m)**	**E_{max}, %DA (±s.e.m)**	**pEC50 (±s.e.m)**	**E_{max}, %DA (±s.e.m)**	**pEC50 (±s.e.m)**	**E_{max}, %DA (±s.e.m)**
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	na		na		na		**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	na		na		na		**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)	na		**7.83** (0.30)	**21** (2)	**7.99** (0.21)	**51** (1)

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[^{35}S]GTPγS binding studies were performed, as in Methods, on membranes of HEK293 cells transfected to transiently express each of the D2L-G protein fusions. Estimates of pEC_{50} for each ligand as well as agonist efficacy measurements relative to dopamine, each (+/- S.E.M.) are provided. na = no detectable stimulation.

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Table 3

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

Receptor:G-protein fusion	**D_{2l} $G\alpha_{i1}$**		**D_{2l} $G\alpha_{i2}$**		**D_{2l} $G\alpha_{i3}$**		**D_{2l} $G\alpha_{o1}$**	
	Dopamine	**S-(-)-3-PPP**	**Dopamine**	**S-(-)-3-PPP**	**Dopamine**	**S-(-)-3-PPP**	**Dopamine**	**S-(-)-3-PPP**
pK_h (±SEM)	**7.12** (0.11)		**7.20** (0.12)		**7.52** (0.13)		**7.67** (0.35)	**8.44** (0.15)
pK_l (±SEM)	**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} (±SEM)	**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)

Membranes from HEK293 cells transfected to express each of the D2L-G protein fusions transiently were used in competition binding studies employing [^{3}H]spiperone and varying concentrartions of either dopamine or S-(-)-3PPP . Data were analyzed as in Methods. pK 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 where 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. form a minimum of three independent experiments.

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1μg/ml Tetracycline	**-**		**+**	
	[^{3}H] Spiperone Binding			
Cell Line	**B_{max}, fMol.mg^{-1}** (± S.E.M.)	**K_d, nM** (± S.E.M.)	**B_{max}, fMol.mg^{-1}** (± S.E.M.)	**K_d, nM** (± S.E.M.)
D_{2l} + Gα_{i1}	**988** (108)	**0.02** (0.01)	**1156** (261)	**0.03** (0.01)
D_{2l} + Gα_{i2}	**1448** (191)	**0.02** (0.01)	**1861** (225)	**0.04** (0.01)
D_{2l} + Gα_{i3}	**538** (52)	**0.03** (0.01)	**510** (120)	**0.02** (0.02)
D_{2l} + Gα_{o1}	**3790** (396)	**0.06** (0.02)	**3864** (249)	**0.07** (0.02)

Table 4 Expression levels of the D2L receptor are unaffected by expression of various G proteins

Flp-In-T-REx HEK293 cells were established able to express pertusiss toxin-resistant forms of each of Gα_{i1}, Gα_{i2}, Gα_{i3} and Gα_{o1} in a tetracycline-dependent fashion (see Figure 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 (-) 1μg/ml tetracycline, saturation [^{3}H]spiperone ligand binding studies were performed on membrane preparation as detailed in Methods. Data represent Means (+/-) S.E.M. of both B_{max} and K_d from at least three experiments performed on different membrane preparations. Induction of G protein expression did not significantly alter D2L receptor expression levels.

Figure 1

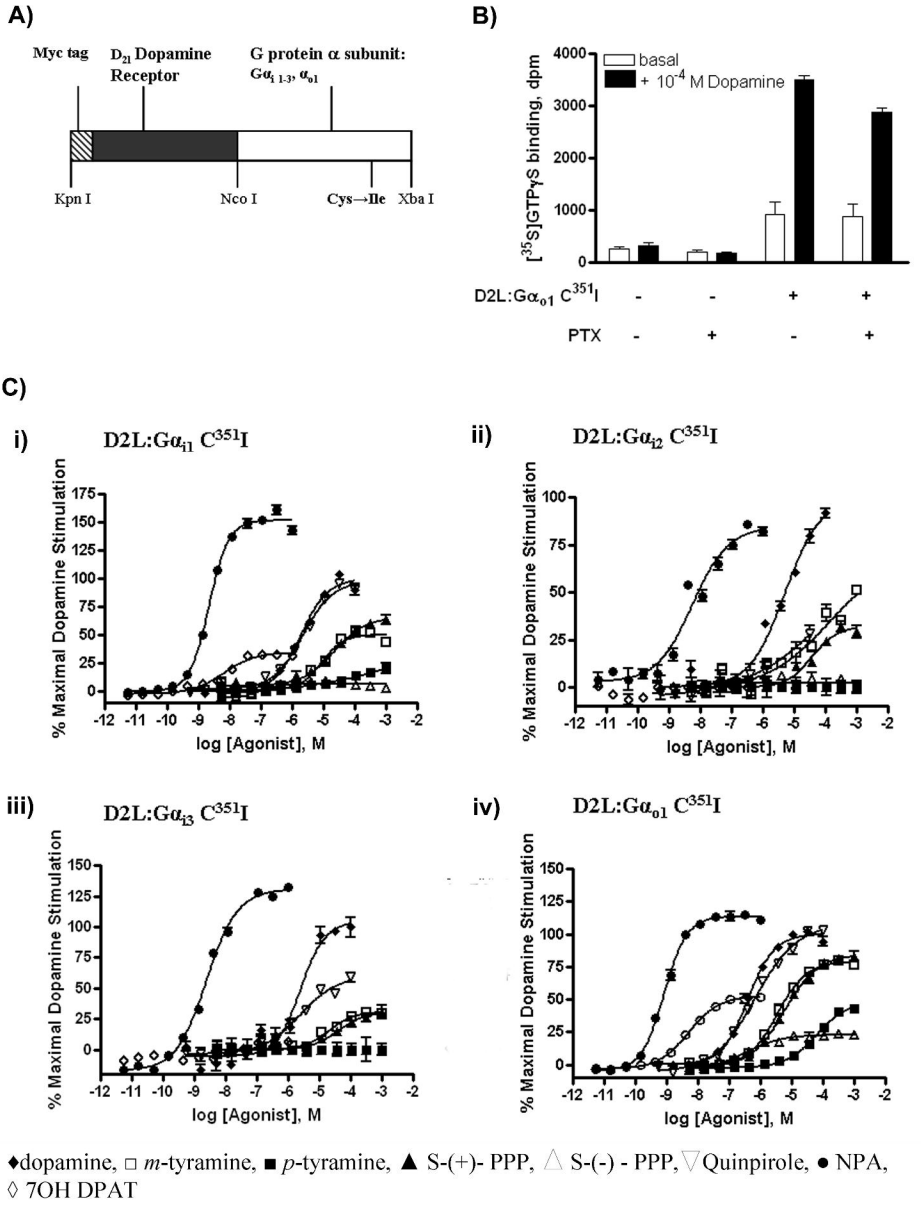

A)
Myc tag
D21 Dopamine Receptor
G protein α subunit: Gαi 1-3, αo1
Kpn I
Nco I
Cys→Ile
Xba I
B)
basal
+ 10-4 M Dopamine
[35S]GTPγS binding, dpm
4000
3000
2000
1000
0
D2L:Gαo1 C351I - - + +
PTX - + - +
C)
i) D2L:Gαi1 C351I
ii) D2L:Gαi2 C351I
iii) D2L:Gαi3 C351I
iv) D2L:Gαo1 C351I
% Maximal Dopamine Stimulation
log [Agonist], M
♦dopamine, □ m-tyramine, ■ p-tyramine, ▲ S-(+)- PPP, △ S-(-) - PPP, ▽ Quinpirole, ● NPA, ◊ 7OH DPAT

Figure 2

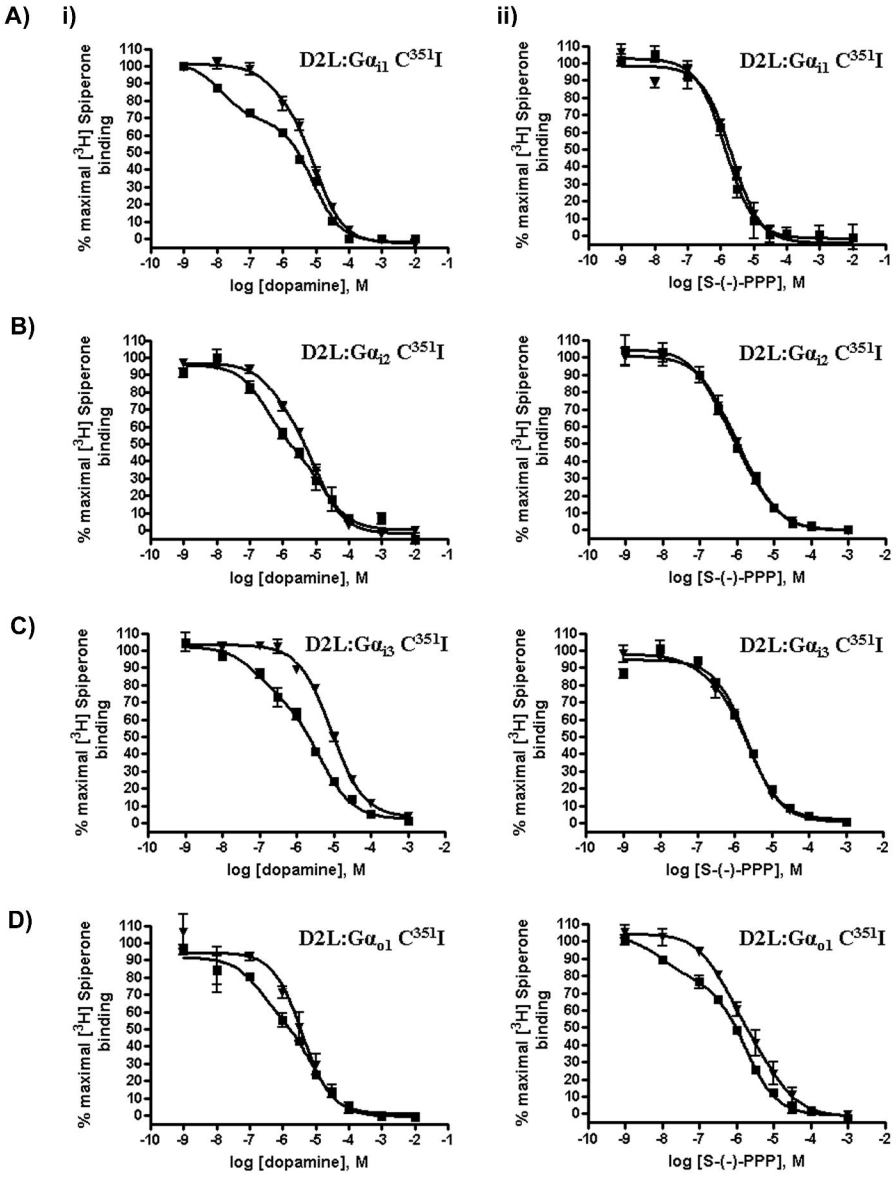

A)
i)
ii)
D2L:Gαi1 C351I
% maximal [3H] Spiperone binding
log [dopamine], M
log [S-(-)-PPP], M
B)
D2L:Gαi2 C351I
C)
D2L:Gαi3 C351I
D)
D2L:Gαo1 C351I

Figure 3

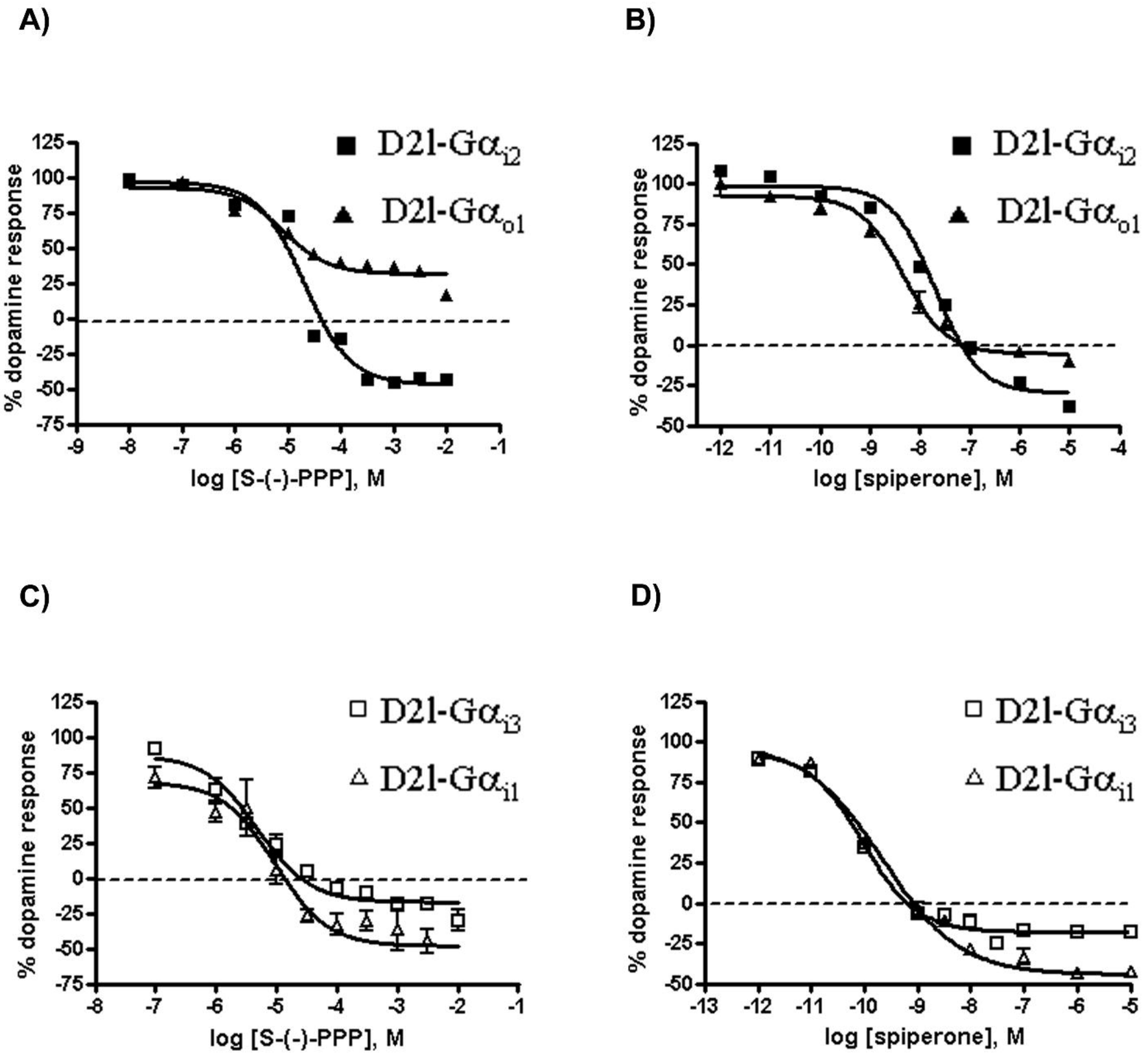
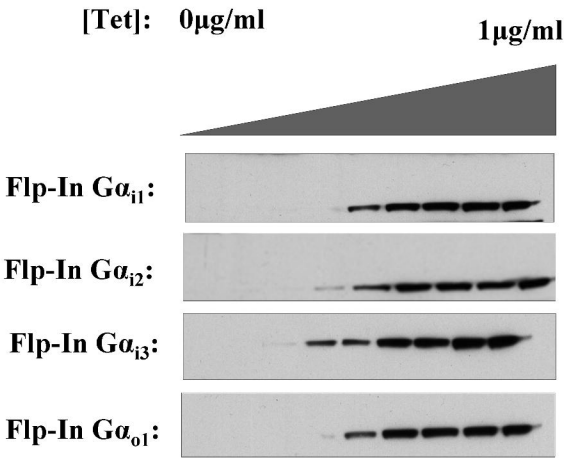

A)
B)
C)
D)
% dopamine response
log [S-(-)-PPP], M
log [spiperone], M
D2l-Gαi2
D2l-Gαo1
D2l-Gαi3
D2l-Gαi1

Figure 4


[Tet]: 0µg/ml
1µg/ml
Flp-In Gαi1:
Flp-In Gαi2:
Flp-In Gαi3:
Flp-In Gαo1:

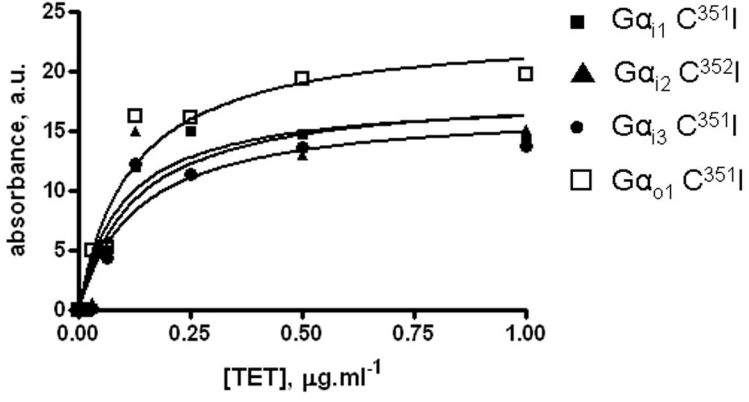
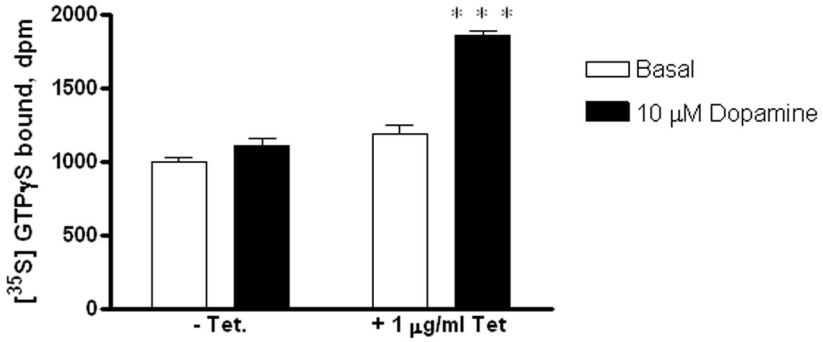

absorbance, a.u.
25
20
15
10
5
0
0.00
0.25
0.50
0.75
1.00
[TET], µg.ml-1
Gαi1 C351I
Gαi2 C352I
Gαi3 C351I
Gαo1 C351I

Figure 5

A)


[35S] GTPγS bound, dpm
2000
1500
1000
500
0
* * *
- Tet.
+ 1 μg/ml Tet
Basal
10 μM Dopamine

B)

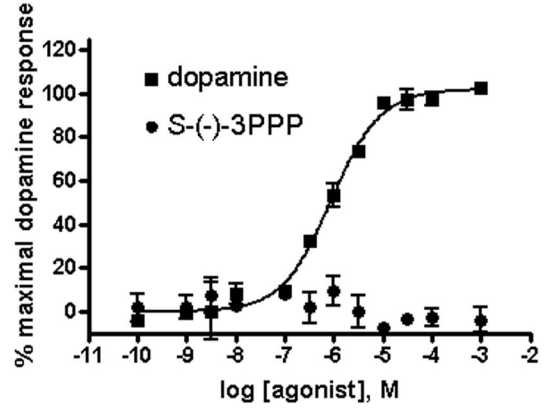
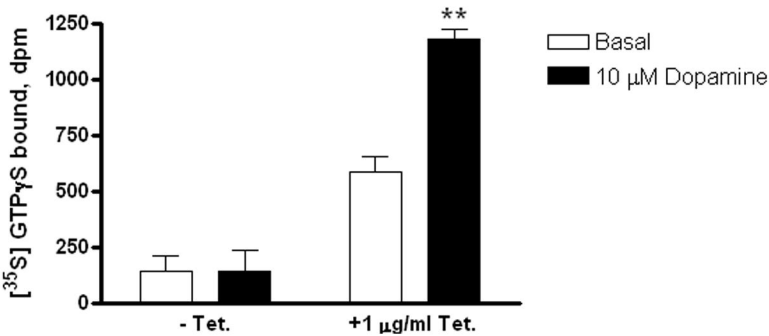

% maximal dopamine response
120
100
80
60
40
20
0
dopamine
S-(-)-3PPP
-11
-10
-9
-8
-7
-6
-5
-4
-3
-2
log [agonist], M

Figure 6

A)


[35S] GTPγS bound, dpm
1250
1000
750
500
250
0
**
- Tet.
+1 μg/ml Tet.
Basal
10 μM Dopamine

B)

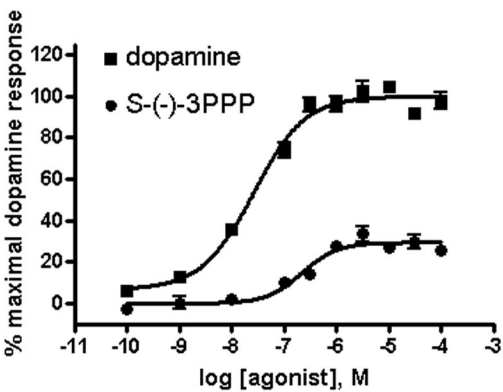
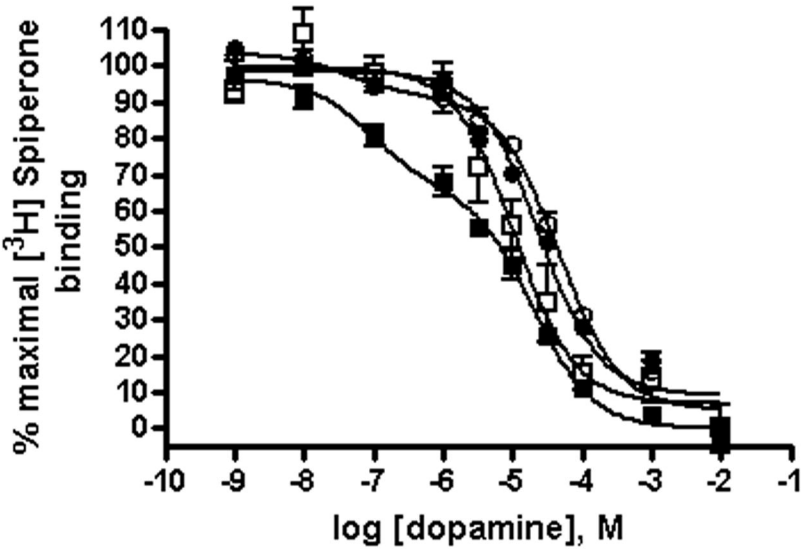

% maximal dopamine response
120
100
80
60
40
20
0
dopamine
S-(-)-3PPP
-11
-10
-9
-8
-7
-6
-5
-4
-3
log [agonist], M

Figure 7

A)


% maximal [3H] Spiperone binding
110
100
90
80
70
60
50
40
30
20
10
0
-10
-9
-8
-7
-6
-5
-4
-3
-2
-1
log [dopamine], M

B)

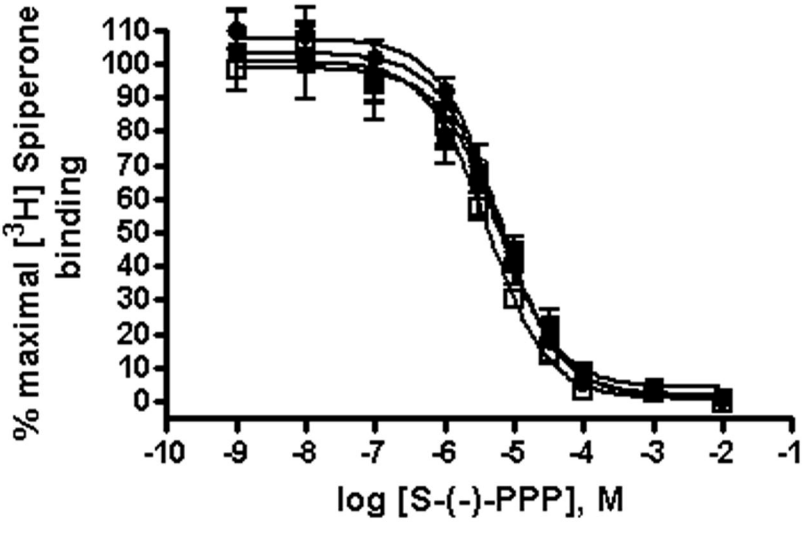
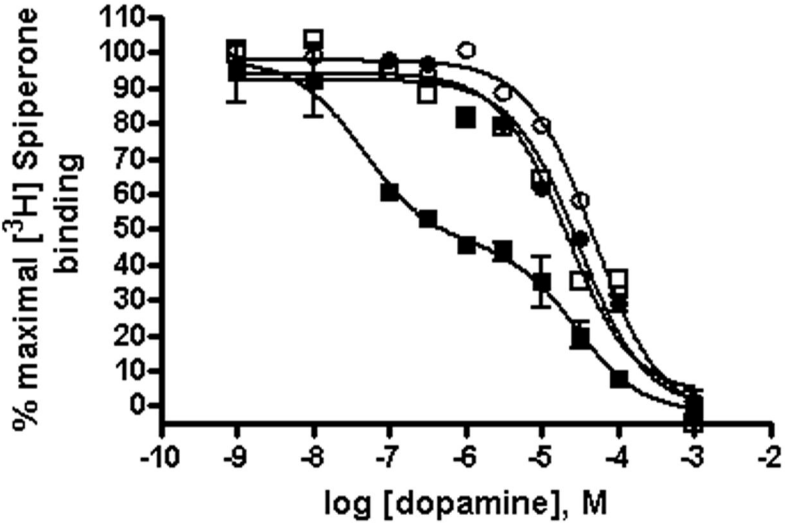

% maximal [3H] Spiperone binding
110
100
90
80
70
60
50
40
30
20
10
0
-10
-9
-8
-7
-6
-5
-4
-3
-2
-1
log [S-(-)-PPP], M

Figure 8

A)


% maximal [3H] Spiperone binding
110
100
90
80
70
60
50
40
30
20
10
0
-10
-9
-8
-7
-6
-5
-4
-3
-2
log [dopamine], M

B)

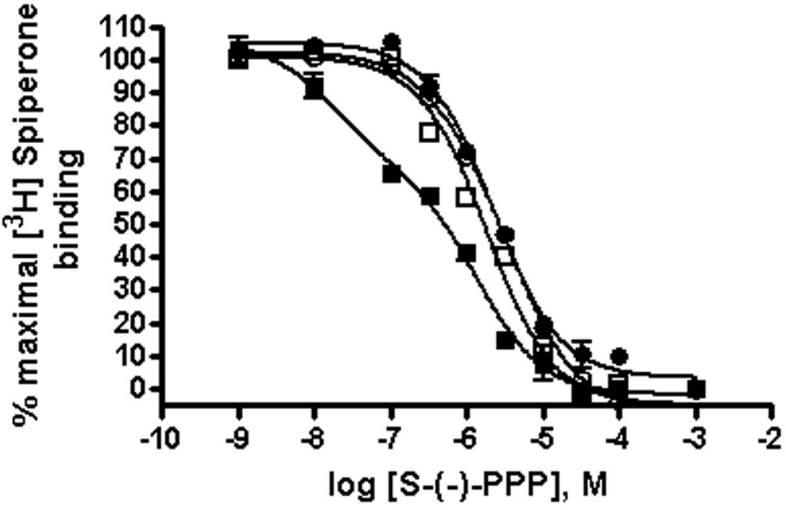

% maximal [3H] Spiperone binding
110
100
90
80
70
60
50
40
30
20
10
0
-10
-9
-8
-7
-6
-5
-4
-3
-2
log [S-(-)-PPP], M

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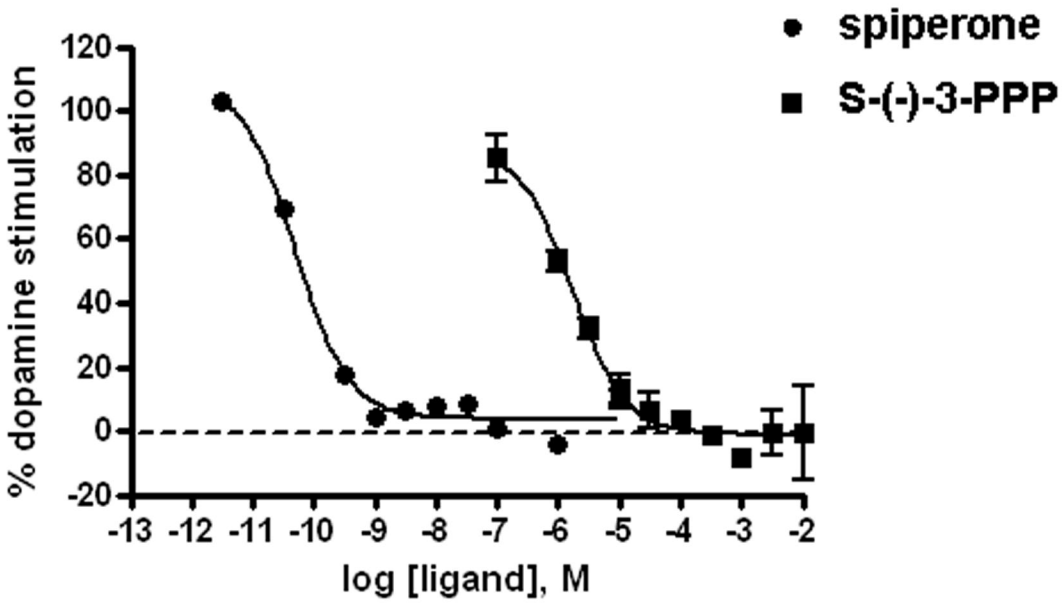
Figure 9

A)



B)