Agonist and antagonist properties of antipsychotics at human dopamine D\textsubscript{4.4} receptors: G-protein activation and K\textsuperscript{+} channel modulation in transfected cells

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Abstract
Interaction at dopamine D\textsubscript{4} receptors may improve cognitive function, which is highly impaired in individuals with schizophrenia, but comparative studies of recent antipsychotics in cellular models of D\textsubscript{4} receptor activation are lacking. Here, we report the in-vitro profile of over 30 ligands at recombinant hD\textsubscript{4.4} receptors. In [\textsuperscript{35}S]GTP\textsubscript{S} binding experiments using membranes of CHO-hD\textsubscript{4.4} cells, apomorphine, preclamol and the selective D\textsubscript{4} agonists, ABT724, CP226269, Ro-10-5824 and PD168077, behaved as partial agonists (E\textsubscript{max} 20–60\% vs. dopamine), whereas L745870 and RBI257, displayed antagonist properties. The ‘conventional’ antipsychotic, haloperidol and the ‘atypicals’, clozapine and risperidone, exhibited antagonist properties, while ‘third generation’ compounds bifeprunox, SLV313 and F15063, acted as partial agonists (10–30\%). Aripiprazole and SSR181507 slightly stimulated [\textsuperscript{35}S]GTP\textsubscript{S} binding at micromolar concentrations. In \textit{Xenopus laevis} oocytes co-expressing hD\textsubscript{4.4} receptors with G-protein-coupled inwardly rectifying potassium (GIRK) channels, apomorphine, preclamol, ABT724, CP226269, and PD168077 stimulated GIRK currents (E\textsubscript{max} 70–80\%). The 5-HT\textsubscript{1A} receptor ligands, WAY100635 and flibanserin, also exhibited partial agonist activity (30\% and 15\%, respectively). Haloperidol, clozapine, olanzapine and nemonapride did not stimulate GIRK currents, whereas aripiprazole, bifeprunox, SLV313 and F15063, but not SSR181507, exhibited partial agonism (E\textsubscript{max} 20–35\%). In-vitro responses depended on experimental conditions: increasing NaCl concentration (30 m M to 100 m M) reduced agonist efficacy in [\textsuperscript{35}S]GTP\textsubscript{S} binding, whereas decreasing the amount of hD\textsubscript{4.4} cRNA injected into oocytes (from 2.0 to 0.5 ng/oocyte) reduced agonist efficacy of several compounds. These data indicate that, unlike conventional or ‘atypical’ antipsychotics, several ‘third generation’ agents display D\textsubscript{4} receptor partial agonism that may be sufficient to influence physiological D\textsubscript{4} receptor activity in vivo.

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Introduction
Schizophrenia patients suffer from a panoply of symptoms, clinically differentiated into positive and negative symptoms as well as cognitive deficits that are only partially responsive to current antipsychotic therapies. In particular, cognitive dysfunction (working and reference memory deficits, perseveration, decreased vigilance, impaired executive function) represents an obstacle to functional recovery and social integration. Whilst conventional antipsychotics such as haloperidol, acting primarily via blockade of dopamine D\textsubscript{2}/D\textsubscript{3} receptors, alleviate positive symptoms, they fail to counter negative symptoms or cognitive deficits (Goldberg and Green, 2002; Harvey and Davidson, 2002; Meltzer et al., 1999). ‘Atypical’ antipsychotics, such as clozapine, olanzapine and risperidone, antagonize D\textsubscript{2/3} receptors but also act at a variety of other receptors, including
several serotonin receptor subtypes. Indeed, combined blockade of D2 and 5-HT1A receptors is associated with reduced extrapyramidal symptoms and increased frontal cortex dopamine levels, a marker of efficacy against negative symptoms/cognitive deficits (Meltzer et al., 2003).

Recently, a ‘third generation’ of potential antipsychotics has attracted increasing attention, by targeting D2-like receptors as well as acting as serotonin 5-HT1A receptor agonists. Such drugs include aripiprazole, bifeprunox, SSR181507, SLV313, RGH188 and FI5063 (Claustre et al., 2003; Kiss et al., 2006; McCreary et al., 2007; Newman-Tancredi et al., 2007; Shapiro et al., 2003; Wadenberg, 2007). Previous studies have demonstrated that such compounds exhibit varying levels of antagonist or partial agonist properties at D2 receptors in vitro in measures of G-protein activation at hD2L receptors (Cosi et al., 2006), phosphorylation of extracellular-regulated kinase (ERK1/2) phosphorylation at hD2S receptors (Bruins Slot et al., 2006), and activation of G-protein-coupled inwardly rectifying potassium (GIRK) currents in Xenopus oocytes transfected with hD2L receptors (Heusler et al., 2007). Further, third-generation antipsychotics also display diverse influence on activation of D3 receptors (Bruins Slot et al., 2007).

Many antipsychotics also bind with high affinity to D4 receptors (Leyesen, 2000; Newman-Tancredi et al., 1997a; Van Tol et al., 1991). Indeed, interest in this receptor subtype was stimulated by the observation that clozapine has slightly higher affinity, and antagonist properties, at this receptor than for D2 or D3 receptors (Leyesen, 2000; Van Tol et al., 1991), suggesting that aspects of clozapine’s ‘atypical’ profile may be attributed to actions at the D4 receptor. However, clinical trials showed that positive symptoms of acutely psychotic schizophrenics were not diminished by selective D4 receptor antagonists (Bristow et al., 1997; Corrigan et al., 2004) or combined D4/5-HT1A antagonism (Truffinet et al., 1999). Thus, the role of D4 receptor interaction in the profile of antipsychotics remains open to discussion and recent evidence from rodent pharmacological models has pointed to the involvement of D4 receptors in the regulation of cognition:

(i) D4 receptors are preferentially localized in brain regions associated with motor or endocrine side-effects, such as the basal ganglia or pituitary gland (De la Garza and Madras, 2000; Meador-Woodruff et al., 1996; Primus et al., 1997; Wong and Van Tol, 2003).

(ii) Electrophysiological studies have demonstrated that D4 receptor activation decreases NMDA receptor-mediated currents in cultures of prefrontal cortex (PFC) neurons and favours NMDA receptor internalization (Wang et al., 2003), consistent with an influence on a brain region of key importance in cognitive function. The same authors also showed that in-vivo treatment of rats with phencyclidine (PCP), a drug that provokes psychotic-like symptoms in humans, disrupts D4 receptor control of NMDA receptor-mediated currents, an effect that could be reversed by clozapine (Wang et al., 2005).

(iii) D4 receptor blockade has protective properties on cultured hippocampal neurons (Bastianetto et al., 2006). In comparison, D4 receptor activation reduces oxidative stress induced in rat cortical neurons (Ishige et al., 2001). Thus, an intermediate degree of partial agonist activity may provide a beneficial influence on preservation of neuronal integrity.

(iv) Both D4 agonists and antagonists are reported to have beneficial effects on aspects of memory/cognition. Thus, in rodents, D4 receptor activation is implicated in memory consolidation (Bernaerts and Tirelli, 2003), short-term social memory (Browman et al., 2005) and increased exploratory activity (Powell et al., 2003). In contrast, in tests of cognition in primates, D4 receptor antagonists opposed the deficits in frontal cortex-dependent working-memory tasks induced by stress (Arnsten et al., 2000) or chronic treatment with PCP (Jentsch et al., 1999). The issue of the balance of activation or blockade of D4 receptors necessary for beneficial properties on cognition was discussed by Zhang et al. (2004) who observed that a D4 receptor antagonist, L745870, had differential effects in rats with high vs. low basal memory performance, suggesting that optimal responses may be obtained with a partial agonist.

(v) D4 receptor knock-out mice exhibit hypersensitivity to psychostimulants (Rubinstein et al., 1997) and D4 receptors are also implicated in deficits in attentional processing, with gene association studies showing that D4 gene expression is associated with attention deficit hyperactivity disorder (ADHD; Faraone and Khan, 2006; Waldman and
Gizer, 2006). Thus, targeting of D₄ receptors provides a potential strategy to manage ADHD, possibly via inhibition of GABA release in the subthalamic nucleus (Floran et al., 2004). In rats with neonatal 6-hydroxydopamine lesions, an animal model of ADHD, a D₄ antagonist attenuated whereas a D₄ agonist accentuated the hyperactivity observed in adulthood (Avale et al., 2004; Zhang et al., 2001).

(vi) In humans, D₄ receptors are up-regulated in schizophrenics, independently of their antipsychotic treatment, suggesting that the changes are disease-related (Lahti et al., 1998). D₄ receptor activation is also relevant to the treatment of sexual dysfunction, a frequently reported side-effect of antipsychotic treatment that can seriously influence treatment outcome in schizophrenia patients (Chue, 2006; Üçok et al., 2007). Compounds possessing D₄ agonist properties, such as ABT724 and flibanserin (a compound also possessing 5-HT₆ receptor agonist activity), are in clinical evaluation for sexual dysfunction (Borsini et al., 2002; Brioni and Moreland, 2006).

Taken together, these observations suggest that a modest level of D₄ receptor activation may be a desirable property for novel antipsychotic agents with improved efficacy against negative and cognitive symptoms of schizophrenia. However, there are no studies, to our knowledge, which provide a comparative analysis of the in-vitro efficacy at dopamine D₄ receptors of established and ‘third-generation’ antipsychotics with combined D₂-like and 5-HT₁A receptor properties. The present work therefore compared the agonist/antagonist properties of a series of antipsychotics at D₄ receptors in vitro in two different systems. First, in a G-protein activation test, using [³⁵S]GTPγS binding as a measure of efficacy. Second, in a GIRK current assay in Xenopus oocytes expressing D₄ receptors. Some of the data presented herein have been reported as an abstract (Cussac et al., 2006).

**Methods**

**Binding affinity at dopamine hD₄,4 receptors**

The D₄ isoform (four-repeat sequence) of D₄ receptors was examined in the present study because it is the most widely expressed allele in humans (Chang et al., 1996). Affinity (inhibition constant, Kᵢ) at hD₄,4 receptors was determined in [³⁵S]pireperone competition-binding experiments. Membranes (21 µg protein per point) from transfected CHO cells stably expressing the human dopamine D₄,4 receptor (PerkinElmer, France) were incubated with [³⁵S]pireperone (0.6 nM) at 37 °C for 120 min in a buffer containing 20 mM Hapes (pH 7.4), 120 mM NaCl, 5 mM KCl, 1 mM EDTA and 5 mM MgCl₂. Incubations were terminated by rapid filtration and radioactivity retained on the filter plates was determined by scintillation counting. Non-specific binding was defined with L745870 (1 µM). Isotherms were analysed by nonlinear regression using the program Prism (GraphPad Software, San Diego, CA) to yield IC₅₀ values. Inhibition constants (Kᵢ) were derived from IC₅₀ values according to the Cheng–Prusoff equation: 

\[ Kᵢ = IC₅₀/(1 + L/K_d) \]

where L is the concentration of [³⁵S]pireperone and Kᵢ is its dissociation constant at hD₄,4 receptors (0.14 nM).

**G-protein activation at hD₄,4 receptors**

Receptor-linked G-protein activation at hD₄,4 receptors was determined by measuring the stimulation of [³⁵S]GTPγS (> 1000 Ci/mmol; GE Healthcare Europe GmbH, Orsay, France) binding essentially as described (Newman-Tancredi et al., 1997a). CHO-D₄,4 membranes (40µg protein per point) were pre-incubated with the compounds for 30 min at 22 °C in a buffer containing 20 mM Hapes (pH 7.4), 3 µM GDP, 3 mM MgCl₂, 100 or 30 mM NaCl. The incubation was started by addition of [³⁵S]GTPγS (0.1 nM) and reagents were incubated for 60 min at 22 °C before rapid filtration and scintillation counting. Non-specific binding was defined with GTPγS (10 µM). Efficacy of agonists was expressed in comparison with that of dopamine (10 µM) that was tested in parallel in each experiment and defined as 100% (basal activity was defined as 0%). In the case of antagonist experiments, the compounds were pre-incubated with CHO-hD₄,4 membranes, as above, before addition of dopamine (1 µM) and [³⁵S]GTPγS. Incubations lasted 60 min at 22 °C, as above. Isotherms were analysed by nonlinear regression to yield pEC₅₀ and E_max values using Prism. For antagonist experiments, Kᵢ values were calculated from IC₅₀ values as follows: 

\[ Kᵢ = IC₅₀/(1 + [Ago]/EC₅₀×Ag₉) \]

where Ago is the concentration of dopamine (1 µM) and EC₅₀×Ag₉ is the EC₅₀ of dopamine for stimulation of [³⁵S]GTPγS binding at hD₄,4 receptors (85 nM with 30 mM NaCl and 160 nM with 100 mM NaCl). Data are presented as means ± S.E.M.

**Xenopus laevis oocyte expression**

The plasmid containing the coding sequence for the human D₄,4 receptor was subcloned into the Xenopus
high expression vector pGEMHE (Liman et al., 1992) and designated pGEMHE/hD4. Plasmids pSP/GIRK1 and pBScMXT/GIRK2 encoding the GIRK1 and GIRK2 potassium channel subunits were prepared as described (Heusler et al., 2005). Plasmids were linearized with NheI (pGEMHE/hD4), EcoRI (pSP/GIRK1) or SalI (pBScMXT/GIRK2) and in vitro transription of RNA was performed using the T7 (pGEMHE/hD4), SP6 (pSP/GIRK1) or T3 (pBScMXT/GIRK2) mMessage mMachine transcription kit (Ambion, Austin, TX). RNA was purified using the RNeasy RNA cleanup kit (Qiagen, Courtaboeuf, France), quantified spectrophotometrically, diluted to the appropriate concentration in RNAse-free water and stored at −80 °C prior to use.

Isolation and separation of oocytes was performed as previously described (Heusler et al., 2005). Defolliculated oocytes were injected with ~50 nl of cRNA solution containing the cRNAs coding for the GIRK1 and GIRK2 channel subunits at a concentration of 20–50 pg cRNA/oocyte each with or without addition of hD4 receptor RNA at 0.5 or 2 ng/oocyte (no measurable dopamine-induced currents were observed in oocytes injected with 2 ng hD4 receptor RNA alone; not shown). After injection, oocytes were kept at 17 °C in ND96 solution [96 mM NaCl, 2 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2, 5 mM Hepes (pH 7.5) with NaOH] supplemented with 50 μg/ml gentamicin. Animals were handled and cared for in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and the European Directive 86/609/EEC, and the protocols were carried out in compliance with French regulations and with local Ethical Committee guidelines for animal research (protocol approved by the Ethics Committee under number 242).

**GIRK current recordings**

Whole-cell oocyte currents were recorded with the two-electrode voltage-clamp technique using a Geneclamp 500 amplifier (Axon Instruments, Union City, CA, USA), as described previously (Heusler et al., 2005). Briefly, oocytes were placed in a recording chamber where they were continuously superfused with ND96 solution (2.5–3.5 ml/min). GIRK currents were recorded in high-potassium solution (hK, similar to ND96, but containing 96 mM KCl, 2 mM NaCl) at a holding potential of −70 mV. D4 receptor ligands dissolved in hK were applied by superfusion. All ligands were applied at a concentration of 1 μM except for aripiprazole and ABT724 (10 μM), in view of their only modest affinity at D4 receptors. At the end of each experiment, BaCl2 (1 mM, dissolved in hK) was applied to quantify the GIRK-independent currents in hK. Evaluation of receptor-independent effects of ligands on GIRK currents was performed as previously described (Heusler et al., 2005). For drugs exhibiting receptor-independent GIRK current inhibitions in this assay, ligand efficacy values in the assay on receptor activation were corrected for the respective value. The pClamp 8 software (Axon Instruments) was used for data acquisition.

**Drugs**

The following compounds were obtained commercially: adrenaline, apomorphine HBr, chlorpromazine, dopamine HCl, L741626 (4-(4-chlorophenyl)-1-(1H-indol-3-yl)methyl)-N-methyl-piperidin-4-ol), haloperidol, noradrenaline, PD168077 (N-[2-cyano-1H-pyrrolizin-1-ylmethyl]-3-methylbenzamide maleate), preclamol, quinolone, raclopide and spiperone, were purchased from Sigma RBI (St Quentin Fallavier, France). Clozapine and L745870 (3-(4-(4-chlorophenyl)piperazin-1-yl)-methyl)-1H-pyrrolo[2,3b]pyridine) were purchased from Tocris (Illkirch, France). ABT724 (2-[(4-pyrindin-2-yl)piperazin-1-yl)methyl]-1H-benzimidazole), amisulpride, aripiprazole, bifeprunox, BP897 (N-4,4,4-2-methoxyphenyl-1-piperazinyl butyl naphthalene-2-carboxamide), CP226269 (5-fluoro-2-(4-pyrindin-2-yl)-piperazin-1-yl)methyl)-1H-indole), fibanserin, F15063 (N-[2,2-dimethyl-2,3-dihydro-benzofuran-7-yloxy)ethyl]-3-(cyclpent-1-enyl)-benzylamine), N-desmethyl-clozapine (NDMC), nemonapride, olanzapine, R1B257 (1,4-iodobenzyl-N-3-isoproxy-2-pyridinyl-N-methylaminopiperidine), Ro-10-5824 (2-methyl-5-((4-phényl-3,6-dihydro-2H-pyrindin-1-ylmethyl)-pyrimidin-4-ylamine), S33084 ((3αR,9β β)-N-[8-(4-chano-1,3a,4,9β-tetrahydro-3H-benzopyran-7-yl)methyl-3-(cyclo pent-1-enyl)-benzylamine), sarizotan HCl, SSR181507 ((3-exo)-8-benzoyl-N-[257-chloro-2,3-dihydro-1,4-benzodioxin-1-yl)methyl]-8 azabicyclo[3.2.1]octane-3-methanamine monohydrochloride), WAY-100635 (N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl-N(N-[2-(4-pyridinyl)cyclohexanecarboxamide trihydrochloride) and ziprasidone were synthesized by Jean-Louis Maurel (Department of Chemistry, Centre de Recherche Pierre Fabre, Castres, France). SLV313 (piperazin-1, 2-(3,3-dihydro-1,4-benzodioxin-5-yl)-4-[5-(4-fluorophenyl)-3-pyridinyl] methhyl) and SLV314 ([(2R)-2H-1,4-benzoxazin-3(4H)-one-8,4-[3-(5-fluoro-1H-indol-3-yl)pro pyl]-1-piperazinyl]-2-methyl) were kindly donated by Solvay Pharmaceuticals (Weesp, The Netherlands).
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Table 1. Influence of neurotransmitters and selective agonists and antagonists on [35S]GTPγS binding to membranes of CHO-hD4, cells

<table>
<thead>
<tr>
<th>Affinity (pKᵢ) binding</th>
<th>G-protein activation (30 mM NaCl)</th>
<th>G-protein activation (100 mM NaCl)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>pEC₅₀</td>
<td>Eₘₐₓ (%)</td>
</tr>
<tr>
<td>Agonists</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dopamine</td>
<td>7.05 ± 0.07 (3)</td>
<td>7.07 ± 0.04 (62)</td>
</tr>
<tr>
<td>Noradrenaline</td>
<td>&lt;5 (3)</td>
<td>5.79 ± 0.07 (3)</td>
</tr>
<tr>
<td>Adrenaline</td>
<td>6.17 ± 0.09 (3)</td>
<td>6.23 ± 0.03 (3)</td>
</tr>
<tr>
<td>Quinoloxane</td>
<td>8.11 ± 0.07 (3)</td>
<td>7.93 ± 0.11 (3)</td>
</tr>
<tr>
<td>Preclamol</td>
<td>5.73 ± 0.02 (3)</td>
<td>5.54 ± 0.21 (3)</td>
</tr>
<tr>
<td>Apomorphine</td>
<td>8.16 ± 0.01 (3)</td>
<td>8.22 ± 0.09 (11)</td>
</tr>
<tr>
<td>PD168077</td>
<td>7.95 ± 0.02 (3)</td>
<td>7.08 ± 0.27 (3)</td>
</tr>
<tr>
<td>ABT724</td>
<td>7.11 ± 0.10 (3)</td>
<td>6.55 ± 0.18 (4)</td>
</tr>
<tr>
<td>CP226269</td>
<td>8.32 ± 0.09 (3)</td>
<td>8.24 ± 0.11 (3)</td>
</tr>
<tr>
<td>Ro-10-5824</td>
<td>7.39 ± 0.10 (3)</td>
<td>7.09 ± 0.39 (3)</td>
</tr>
<tr>
<td>Antagonists</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spiperone</td>
<td>9.54 ± 0.10 (3)</td>
<td>–</td>
</tr>
<tr>
<td>RBI257</td>
<td>8.42 ± 0.08 (3)</td>
<td>–</td>
</tr>
<tr>
<td>L745870</td>
<td>9.15 ± 0.03 (3)</td>
<td>–</td>
</tr>
<tr>
<td>L741,626</td>
<td>6.23 ± 0.04 (3)</td>
<td>–</td>
</tr>
<tr>
<td>S33084</td>
<td>6.24 ± 0.02 (3)</td>
<td>–</td>
</tr>
<tr>
<td>Raclopride (–)</td>
<td>&lt;5 (3)</td>
<td>–</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± s.e.m. (n determinations). Drugs are listed in order of pEC₅₀ with 30 mM NaCl. Binding affinity at hD₄ receptors was determined in competition-binding experiments with [3H]spiperone. Agonist efficacy was determined by incubating (60 min, 22 °C) membranes of CHO stably expressing hD₄ receptors with compounds and [35S]GTPγS (0.1 nM) in buffer containing either 30 mM or 100 mM NaCl to yield pEC₅₀ and Eₘₐₓ values. The latter are expressed as a percentage of the effect of dopamine (10 μM) tested in each experiment. For agonist experiments, dopamine (1 μM) was included in the incubations. Inactive = less than 5% stimulation.

Results

Affinity at hD₄ receptors

Dopamine exhibited modest affinity at hD₄ receptors (pKᵢ=7.05), as previously reported (Newman-Tancredi et al., 1997a). Under present conditions, adrenaline and noradrenaline exhibited low affinities (pKᵢ=6.17 and <5, respectively). Selective dopamine D₄ receptor agonists displayed generally higher affinities with pKᵢ values of 7–8 for PD168077, ABT724 and Ro-10-5824, and of 8.32 for CP226269 (Table 1, first column).

The selective D₄ receptor antagonists, L745870 and RBI257, as well as the non-selective dopaminergic antagonist, spiperone, displayed high pKᵢ values at hD₄ receptors. In contrast, the D₅/D₄ receptor antagonist, raclopride, the D₃ receptor selective antagonist, S33084 and the preferential D₃ receptor antagonist, L741626, exhibited low affinity at hD₄ receptors (pKᵢ values <6.5).

Among the antipsychotics (Table 2, first column), nemonapride exhibited the highest affinity at hD₄ receptors (pKᵢ=9.67) and haloperidol and risperidone displayed marked affinity (pKᵢ values ~8) whereas clozapine and olanzapine were less active (pKᵢ ~7.3). Recent antipsychotics with combined D₂-like and 5-HT₁A receptor activity displayed widely differing affinities at hD₄ receptors. Thus bifeprunox had high affinity (pKᵢ=9.51) whereas aripiprazole was over 100-fold less potent (pKᵢ=7.34). SLV313, SSR181507 and F15063 exhibited intermediate affinity (pKᵢ 8–9). SLV314, a putative antipsychotic that also exhibits serotonin reuptake inhibition activity, showed high affinity (pKᵢ ~9).

The anti-dyskinetic drug, sarizotan, the anti-sexual dysfunction drug, fibanserin, and the D₃ receptor
partial agonist, BP897, exhibited intermediate affinity for hD₄ receptors (pKᵢ, 8.07, 7.31 and 7.24, respectively).

**Efficacy at hD₄ receptors determined by [³⁵S]GTPγS binding**

Using buffer containing 30 mM NaCl (conditions that favour detection of weak partial agonist properties), basal [³⁵S]GTPγS binding amounted to 9700 ± 400 dpm. This increased 2-fold to 19300 ± 800 dpm in the presence of 10 μM dopamine, as tested systematically in all experiments to enable normalization of relative efficacy values. Dopamine, adrenaline, noradrenaline and quinolone fully activated [³⁵S]GTPγS binding to CHO-hD₄ cell membranes (Figure 1, Table 1). In contrast, the other agonists exhibited lower efficacy: preclamol and apomorphine, as well as the selective D₄ agonists, ABT724 and PD168077, behaved as partial agonists (Eₘₐₓ values 37–55% relative to dopamine). Two other selective agonists, Ro-10-5824 and CP226269 exhibited modest efficacy (Eₘₐₓ ≤ 20%).

As expected, the selective D₄ receptor ligands, L745870 and RBL257, and the antipsychotics, clozapine, haloperidol, risperidone and olanzapine, did not stimulate [³⁵S]GTPγS binding (Tables 1 and 2, third column). In contrast, the ‘third-generation’ antipsychotics, bifeprunox, SLV313 and FI5063, as well as SLV314 and BP897, stimulated [³⁵S]GTPγS binding with modest efficacy (Eₘₐₓ values 15–29%; Fig. 2). SSR181507 very weakly stimulated [³⁵S]GTPγS binding (Table 2) whereas no significant stimulation was detected with the clozapine metabolite, NDMC. Aripiprazole stimulated [³⁵S]GTPγS binding at the highest concentration (26% at 10 μM). All the drugs were able to antagonize [³⁵S]GTPγS binding induced by dopamine, demonstrating an interaction at D₄

<table>
<thead>
<tr>
<th>Table 2. Influence of antipsychotics and psychotropic agents on [³⁵S]GTPγS binding to membranes of CHO-hD₄ cells</th>
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<tbody>
<tr>
<td><strong>Affinity</strong> ([³⁵S]spiperone binding)</td>
</tr>
<tr>
<td>pKᵢ</td>
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<tr>
<td>-----------------</td>
</tr>
<tr>
<td>Sarizotan</td>
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<tr>
<td>FI5063*</td>
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<tr>
<td>SLV314</td>
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<tr>
<td>BP897</td>
</tr>
<tr>
<td>Bifeprunox</td>
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<tr>
<td>Filbanserin</td>
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<tr>
<td>SLV313</td>
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<tr>
<td>SSR181507</td>
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<tr>
<td>Aripiprazole</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± S.E.M. (n determinations). Drugs are listed in order of Eₘₐₓ with 30 mM NaCl.

Binding affinity at hD₄ receptors was determined in competition-binding experiments with [³⁵S]spiperone. Agonist efficacy was determined by incubating (60 min, 22°C) membranes of CHO stably expressing hD₄ receptors with compounds and [³⁵S]GTPγS (0.1 μM) in buffer containing either 30 mM or 100 mM NaCl to yield pEC₅₀ and Eₘₐₓ values. The latter are expressed as a percentage of the effect of dopamine (10 μM) tested in each experiment. For antagonist experiments, dopamine (1 μM) was included in the incubations.

Inactive = less than 5% stimulation; n.c., not computable; n.d., not determined.

*Data for FI5063 under 30 mM NaCl conditions is from Newman-Tancredi et al. (2007).

bStimulation observed at maximal concentration tested (10 μM).
receptors in the present cell membrane preparation. Consistent with partial agonist properties, those compounds that showed some efficacy did not reduce dopamine-stimulated $[^{35}]$GTP$\gamma$S binding to basal levels. In contrast, they only reduced $[^{35}]$GTP$\gamma$S binding to the level attained by the partial agonist when tested alone (see Figures 1 and 2). Sarizotan (33%) and flibanserin (16%) exhibited efficacy about two thirds and one third of that of apomorphine, respectively.

The potency ($pEC_{50}$) of agonists for stimulation of $[^{35}]$GTP$\gamma$S binding was correlated with the affinity ($pK_i$) of the ligands in competition-binding experiments (Figure 3a). Likewise, the potency of the antagonists ($pK_B$) for reversal of dopamine-stimulated $[^{35}]$GTP$\gamma$S binding was highly correlated with the affinity ($pK_i$) of the ligands (Figure 3b).

A parallel set of $[^{35}]$GTP$\gamma$S binding experiments was carried out with a higher NaCl concentration (100 mM), similar to conditions reported previously (Newman-Tancredi et al., 1997a). Under these conditions, basal $[^{35}]$GTP$\gamma$S binding amounted to 6400 ± 400 dpm and increased 2.2-fold to 14300 ± 1100 dpm with 10 $\mu$M dopamine. The apparent efficacy of the agonists ($E_{max}$ values relative to dopamine) was lower than that measured using buffer containing 30 mM NaCl. The difference was most marked for ABT724, CP226269 and Ro-10-5824: their $E_{max}$ values were approximately halved under high NaCl conditions (Tables 1 and 2). In comparison, the $E_{max}$ values of adrenaline and noradrenaline were less markedly affected but the partial agonist activity of flibanserin was no longer detectable. The use of high NaCl conditions also reduced $pEC_{50}$ values for several agonists, including CP226269 and Ro-10-5824. In contrast, PD168077 and sarizotan exhibited similar potency under both NaCl conditions (Tables 1 and 2). These data suggest that changes in receptor–G-protein interactions induced by NaCl are agonist-dependant, possibly reflecting ‘signalling specificity’ of the ligands for specific receptor conformations.

Efficacy at $hD_{4.4}$ receptors determined by activation of GIRK currents in Xenopus oocytes

In experiments in which oocytes were injected with 2 ng $hD_{4.4}$ receptor cRNA, application of dopamine (1 $\mu$M) produced an increase in $K^+$ current through co-expressed GIRK channels (Figure 4a). Under these conditions, dopamine exhibited a $pEC_{50}$ of 8.60 ± 0.13 (Fig. 4b).

Substantial partial agonism was revealed for a series of dopaminergic ligands (Figure 5a). Thus,
apomorphine activated GIRK currents to about 80% of the effect of dopamine at 1 mM, whereas preclamol as well as the selective D₄ receptor agonists PD168077, ABI724 and CP226269 all had efficacies of about 70%. It is possible that a higher efficacy of preclamol could have been observed if a higher concentration had been used (e.g. 10 mM instead of 1 mM) but, in any case, it is clear that it has substantial agonist properties in this system. Sarizotan, a 5-HT₁A receptor agonist, and WAY100635, a 5-HT₁A receptor antagonist, induced more modest current activation (≈40% and 30%, respectively). Slight current activation was also observed with flibanserin (≈15%), and L745870 and RBI257 (≈9% and 6%, respectively). In separate experiments, the D₄ receptor antagonist, L745870, antagonized the activation of GIRK currents induced by dopamine and the selective agonist, PD168077. Thus dopamine (10 nM) and PD168077 (100 nM) stimulated GIRK currents by 66.3 ± 9.2% and 46.2 ± 2.6%, respectively, when applied alone, but only by 14.9 ± 5.5% and -2.2 ± 1.2% in the presence of 1 μM L745870 (n = 3 for all values).

As concerns antipsychotics, GIRK current recordings clearly revealed partial agonist properties of FI5063, aripiprazole, SLV313 and bifeprunox (Eₘₐₓ values ≈20–30%), whereas all the other drugs, including haloperidol, clozapine, olanzapine, risperidone, ziprasidone, nemonapride, NDMC and...
SSR181507 were inactive. The current activation induced by F15063 (1 μM), aripiprazole (10 μM), SLV313 (1 μM) and bifeprunox (1 μM) was antagonized by the D4 antagonist, RBI257 (1 μM). Residual stimulation in the presence of 1 μM RBI257 was 1.8 ± 2.4% for F15063, 2.6 ± 1.7% for aripiprazole, 3.3 ± 1.0% for SLV313 and 0.5 ± 0.4% for bifeprunox (n = 4–6). SSR181507 (1 μM), on the other hand, antagonized PD168077 (100 nM)-induced GIRK current activation, thus confirming its antagonist properties at hD4 receptors in this model (PD168077 induced 60.3 ± 7.3% stimulation when applied alone, but only 17.8 ± 2.2% in the presence of SSR181507; n = 7).

The marked activation observed with the dopaminergic agonists (Figure 5a) suggested that relatively high levels of hD4 receptor expression were generated by injecting 2.0 ng cRNA/oocyte. For comparison, a lower (0.5 ng) quantity of cRNA was therefore injected into oocytes, leading to a decrease of the pEC50 for dopamine to 8.03 ± 0.15, consistent with lower hD4 receptor expression. Under these conditions, the relative Emax values for apomorphine and preclamol, as well as for the specific D4 receptor ligands, PD168077, ABT724, CP226269, L745870 and RBI257 were diminished (Figure 5b). In addition, the partial agonist activity of F15063 and bifeprunox was reduced to very low levels and the activity of aripiprazole and SLV313 was no longer detectable.

Discussion

The key finding of the present study is that ‘third-generation’ antipsychotics currently undergoing clinical or pre-clinical characterization display markedly diverse agonist/antagonist properties in cellular models of dopamine D4 receptor activation. This has been demonstrated here for two different measures of D4 receptor signalling: G-protein activation, determined by [35S]GTPyS binding, and coupling to GIRK channels, determined by electrophysiological recordings in Xenopus oocytes.

Influence of dopaminergic ligands on G-protein activation

In measures of G-protein activation (with 30 mM NaCl), dopamine robustly stimulated [35S]GTPyS binding. In confirmation of previous reports (Czermak et al., 2006; Lanau et al., 1997; Newman-Tancredi et al., 1997b), adrenaline and noradrenaline also efficaciously stimulated [35S]GTPyS binding albeit with reduced potency (EC50 values increased by 7- and 19-fold relative to dopamine, respectively). These data indicate that these endogenous agonists are capable of convergent signalling at D4 receptors (see also Wedemeyer et al., 2007 for activation of GIRK by dopamine and noradrenaline at D4 receptors). The profile of action of other ligands was in agreement with their receptor selectivity: the selective D4 receptor agonists, ABT724, CP226269, PD168077 and Ro-10-5824 modestly stimulated [35S]GTPyS binding whereas the antagonists, L745870 and RBI257 did not. The D2/D3 antagonist, raclopride, the preferential D2 antagonist, L741626 and the selective D3 antagonist, S33084 (Millan et al., 2000), were inactive. In contrast, antipsychotics exhibited wide variety of efficacy at D4 receptors. Thus bifeprunox, F15063, SLV313 and BP897 exhibited (modest) partial agonist properties whereas the
established antipsychotics clozapine, haloperidol, olanzapine and risperidone were devoid of agonist properties, as previously reported (Burnstein et al., 2005; Newman-Tancredi et al., 1997a; Patel et al., 2003). Sarizotan, an antidyskinetic agent, and flibanserin, developed as an antidepressant and anti-sexual dysfunction drug (Borsini et al., 2002; Kuzhikandathil and Bartoszyk, 2006), both exhibited partial agonist properties at hD₄ receptors.

It is important to note that the partial agonists were able to antagonize dopamine-stimulated G-protein activation, bringing [³⁵S]GTPγS binding down to the same level as that observed with high concentrations of the drug alone (Figures 1, 2). Physiologically, such compounds have been described as ‘dopamine stabilizers’, activating dopaminergic transmission when it is insufficient and attenuating it when it is excessive (Bolonna and Kerwin, 2005; Yocca and Altar, 2006). A similar ‘stabilizing’ influence may

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**Figure 4.** hD₄ receptor-mediated GIRK current activation by dopamine (DA) in Xenopus oocytes. Oocytes were injected with hD₄ receptor cRNA (2 ng) and GIRK1/2 channel subunit cRNAs (20 pg each). Oocytes were clamped at −70 mV, and dopamine was applied by superfusion for 90 s as indicated by the bar in panel (a). Basal GIRK current was activated by application of high potassium solution (hK, containing 96 mM K⁺), and the level of GIRK-independent potassium current was determined by superfusion with hK solution supplemented with 1 mM BaCl₂. At the beginning and at the end of each experiment, oocytes were kept in ND96 solution. (a) Current trace of GIRK current activation by dopamine (1 µM). (b) Concentration–response curve of dopamine effects, normalized to the mean current evoked by the maximal dose of dopamine (1 µM) in oocytes of the respective batch.

**Figure 5.** Influence of dopaminergic ligands on hD₄ receptor-mediated GIRK current activation in Xenopus oocytes. (a) Currents measured at a high level of receptor expression (2.0 ng cRNA/oocyte). The inset shows the Eₘᵢₓ values determined for a series of agonists in GIRK current and [³⁵S]GTPγS binding experiments (Tables 1 and 2; 30 mM NaCl). The straight line indicates a theoretical correlation with a slope of 1 and passing through zero. The circled points indicate outliers (apomorphine, preclamol, PD168077, ABT724 and CP226269) that exhibit higher efficacy for GIRK current activation than for [³⁵S]GTPγS binding. (b) Currents measured at a lower level of receptor expression (0.5 ng cRNA/oocyte). Data are expressed as percent current stimulation relative to that induced by dopamine (DA) (1 µM). Histograms are mean ± S.E.M. values from 3 to 8 oocytes. All drugs were tested at a concentration of 1 µM except for ABT724 and aripiprazole (10 µM).
apply to D₄ receptors in the areas of cognition/mnesic function increasingly associated with this receptor (see discussion below). However, levels of partial agonism in vitro are highly dependent on experimental conditions, so prudence must be exerted in extrapolating from the present data to physiological responses (cf. Gazi et al., 1999). Thus, when a higher concentration of sodium (100 mM) is used in the incubation buffers for [³⁵S]GTPγS binding, Eₘₐₓ values (relative to dopamine) are decreased and a 3- to 5-fold loss of potency (EC₅₀ values) is seen for most agonists. These data are consistent with the influence of NaCl on opposing receptor–G-protein coupling and therefore decreasing response to agonists (Czermak et al., 2006; Newman-Tancredi et al., 1997a) and raise the issue of the most physiologically relevant conditions in which to undertake in-vitro studies. Indeed, whilst physiological extracellular sodium concentrations are in the 100 mM range, the [³⁵S]GTPγS binding assay reflects GTP/GDP exchange at the Ga subunit of heterotrimeric G-proteins that are located at the intracellular face of plasma membranes where NaCl concentration is much lower (~10 mM), except under neuronal depolarization conditions, when sodium influx transiently increases intracellular NaCl levels. Therefore, a low NaCl concentration may be more ‘physiological’ when measuring G-protein activation, as has been discussed previously for 5-HT₄ receptors (Urban et al., 2007) and, in the present study, it exhibited low potency (Eₘₐₓ 13–32%) will also influence the absolute levels of efficacy for agonists in in-vitro studies, and (ii) raise the issue of the amount of receptor activation necessary for pharmacological actions in vivo. Hence, although the precise receptor densities and G-protein subtypes encountered by D₄ receptors in their neuronal microenvironments are unknown, it is possible that even modest partial agonist properties may be sufficient to exert an influence in vivo. This is confirmed by the observation that amphetamine and ABT724 (Eₘₐₓ 47% and 36%, respectively, Table 1) facilitated sexual activity in rodents (Brioni and Moreland, 2006). Further, PD168077 (Eₘₐₓ 39% in the present system) stimulated ERK phosphorylation in the paraventricular nucleus, a region associated with sexual behaviour (Bitner et al., 2006). It may be surmised, therefore, that compounds such as bifeprunox, sarizotan, SLV313 and F15063, that possess partial agonist properties (Eₘₐₓ 13–32%) will also influence D₄ activity under physiological conditions. Indeed, F15063 and SLV313 reversed scopolamine-induced memory deficits in a rat social recognition paradigm (Bardín et al., 2006; Depoortère et al., 2007), an action that is blocked by pre-treatment with L745870. Thus, D₄ receptor activation by F15063 and SLV313 appears responsible for their activity in a model of cholinergic deficit, a finding in accordance with the known pro-cholinergic action of SLV313 in neurochemical measures (McCreary et al., 2007).

**Actions of agonists and antagonists on GIRK currents in Xenopus oocytes**

In general, the compounds that activated [³⁵S]GTPγS binding to CHO-hD₄ membranes also increased GIRK channel currents in *Xenopus* oocytes, demonstrating that they are capable of regulating both of these transduction systems linked to D₄ receptors (cf. Werner et al., 1996). However, it is interesting to compare in more detail the efficacy of agonists from [³⁵S]GTPγS binding experiments, a measure of activation of G-protein Ga subunits, with efficacy determined in GIRK channel activation experiments, a prototypical measure of Gα subunit activation (Pillai et al., 1998). Whilst apomorphine exhibited high efficacy in both cases, some other drugs exhibited preferential efficacy for one transduction system vs. the other. Hence, sarizotan exhibited substantial partial agonist efficacy for [³⁵S]GTPγS binding (Eₘₐₓ = 32% with 30 mM NaCl conditions), greater than that observed with the selective agonist, CP226289 (Eₘₐₓ = 23%). In contrast, sarizotan was less efficacious for activation of GIRK currents (Eₘₐₓ < 40%) than CP226289 (Eₘₐₓ ~ 70%). These data suggest that CP226289 may preferentially exert its agonist properties via GIRK channels whereas sarizotan may predominantly influence other G-protein activation pathways and indicate that care must be taken in defining the order of efficacy of ligands according to only a single cellular response. Indeed, the concept of ‘functional selectivity’ or ‘agonist-directed trafficking of receptor signalling’ (ADTRS; Kenakin, 2001; Perez and Karnik, 2005) whereby different agonists may preferentially influence specific signalling pathways, is reported at several receptors, including 5-HT₂C and dopamine D₄ (Berg and Clarke, 2006; Lane et al., 2007). Aripiprazole has been reported to exhibit ADTRS at dopamine D₂ receptors (Urban et al., 2007) and, in the present study, it exhibited low potency for stimulation of [³⁵S]GTPγS binding to CHO cell membranes (Table 2) but stimulated GIRK currents to the same extent as bifeprunox or F15063 (Fig. 5).

However, caution must be exercised when interpreting in-vitro functional responses. Indeed, the influence of the cellular background of the cells (CHO vs. oocytes) may play a role in the differences observed between experimental systems. Further, other
authors have reported diverse effects in different functional read-outs. Shapiro et al. (2003) reported that aripiprazole modulated K+ currents and behaved as an efficacious and potent agonist at D4 receptors for inhibition of adenylyl cyclase activity, but did not provide comparative information for other antipsychotics. Similarly, Kuzhikanthathil and Bartoszyk (2006) reported that sarizotan was an efficacious agonist at hD4a (and D4b) receptors for inhibition of adenylyl cyclase and activation of GIRK currents but no other antipsychotics were compared. Taken together, these reports underline the importance of comparative information using a library of reference compounds in order to ‘calibrate’ in-vitro functional responses.

It is interesting to note that the selective D4 receptor ligand, L745870, exhibited slight stimulation of GIRK currents, whereas no agonist activity was detectable in [35S]GTPγS binding experiments. Previous studies of the efficacy of L745870 have indicated that it behaves as an antagonist or weak partial agonist depending on the cellular system adopted (Gazi et al., 1999; Newman-Tancredi et al., 1997a; Patel et al., 2003; Pillai et al., 1998). The present data therefore support the notion that this ‘antagonist’ actually expresses weak efficacy at D4 receptors. Similarly, the selective 5-HT1A receptor antagonist, WAY100635, also stimulated GIRK currents, indicating that it can act as a D4 receptor agonist, as previously reported (Chemel et al., 2006), although its efficacy and potency are modest (Figure 5; Martel et al., In Press). Finally, it should be noted that, as in the case of [35S]GTPγS binding studies, experimental conditions are a crucial factor in assessing agonist efficacy. Thus, the amount of cRNA used to transfect the oocytes directly influenced the relative stimulation of GIRK currents, presumably by modifying the amount of D4 receptor expressed at the cell surface.

Conclusions
The present study demonstrates that a range of dopaminergic ligands and antipsychotics act with differing agonist or antagonist properties at recombinant human D4 receptors. The capacity to activate D4 receptors in vitro is dependent on experimental conditions such as sodium concentrations for [35S]GTPγS binding and the quantity of transfected cRNA for GIRK currents in oocytes. Thus, D4 receptor stimulation may be sensitive to increases in receptor expression, for example in conditions of pathological up-regulation, as has been observed in post-mortem brain from schizophrenics (Lahti et al., 1998). Generally, compounds that activate [35S]GTPγS binding also increase GIRK currents although sarizotan preferentially activates [35S]GTPγS binding whereas CP226269 preferentially stimulates GIRK currents.

Extrapolations from the present data to in vivo and/or therapeutic significance should be made with caution, in view of the diverse activity of antipsychotics at multiple receptor subtypes. Nevertheless, whereas older antipsychotics, such as haloperidol, clozapine and risperidone, act as antagonists at D4 receptors, several ‘third-generation’ ligands such as bifeprunox, SLV313, F15063 and aripiprazole (at high concentrations) but not SSR181507, exhibit marked partial agonist activity. Their efficacy corresponds to about one to two thirds of that of apomorphine, ABT724 and PD168077, compounds that have known in-vivo D4 receptor activity in rodent models of ERK1/2 activation and sexual activity (Bitner et al., 2006; Brioni and Moreland, 2006). Further, the activity of F15063 and SLV313 in a model of memory deficit is blocked by L745870 (Bardin et al., 2006; Depoortere et al., 2007). It may therefore be concluded that even modest in-vitro partial agonist properties will translate in vivo to detectable physiological activity, particularly in regards to cognitive profile (see Introduction) and/or influence on sexual dysfunction.

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All authors are employees of the Pierre Fabre Research Centre, Castres, France.

References
Bardin L, Newman-Tancredi A, Depoortere R (2006). Effects of novel antipsychotics with preferential activity at D2-like and 5-HT1A receptors, in comparison with typical and


**Lane JR, Powney B, Wise A, Rees S, Milligan G** (2007). Protean agonism at the dopamine D₂ receptor: S-3-(3-


**Newman-Tancredi A, Audinot V, Gobert A, Millan MJ** (1997b). Noradrenaline and adrenaline are high affinity...


