

RESEARCH PAPER

Rotigotine is a potent agonist at dopamine D₁ receptors as well as at dopamine D₂ and D₃ receptors

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Received

19 September 2014

Revised

10 October 2014

Accepted

14 October 2014

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BACKGROUND AND PURPOSE

Rotigotine acts as a dopamine receptor agonist with high affinity for the dopamine D₂, D₃, D₄ and D₅ receptors but with a low affinity for the dopamine D₁ receptor. We have investigated this further in radioligand binding and functional studies and compared the profile of rotigotine with that of other drugs used in the treatment of Parkinson's disease (PD).

EXPERIMENTAL APPROACH

The binding of rotigotine to human dopamine D₁, D₂, D₃, D₄ and D₅ receptors was determined in radioligand binding studies using [³H]rotigotine and compared with that of standard antagonist radioligands. Functional interactions of rotigotine with human dopamine receptors was also determined.

KEY RESULTS

[³H]rotigotine can be used as an agonist radioligand to label all dopamine receptor subtypes and this can be important to derive agonist affinity estimates. Rotigotine maintains this high affinity in functional studies at all dopamine receptors especially D₁, D₂ and D₃ receptors and, to a lesser extent, D₄ and D₅ receptors. Rotigotine, like apomorphine but unlike ropinirole and pramipexole, was a potent agonist at all dopamine receptors.

CONCLUSIONS AND IMPLICATIONS

Rotigotine is a high-potency agonist at human dopamine D₁, D₂ and D₃ receptors with a lower potency at D₄ and D₅ receptors. These studies differentiate rotigotine from conventional dopamine D₂ agonists, used in the treatment of PD, such as ropinirole and pramipexole which lack activity at the D₁ and D₅ receptors, but resembles that of apomorphine which has greater efficacy in PD than other dopamine agonists but has suboptimal pharmacokinetic properties.

Abbreviations

(-)-3-PPP, (-)-3-(3-hydroxyphenyl)-N-n-piperidine; CDS, cellular dielectric spectroscopy; GppNHp, 5'-guanylyl imidodiphosphate; HBSS, Hank's balanced salt solution; PD, Parkinson's disease

Tables of Links

| TARGETS |
|-----------------------------------|
| Dopamine D ₁ receptors |
| Dopamine D ₂ receptors |
| Dopamine D ₃ receptors |
| Dopamine D ₄ receptors |
| Dopamine D ₅ receptors |

| LIGANDS | |
|-----------------------------|-------------|
| [³ H]raclopride | Dopamine |
| [³ H]SCH23390 | Lisuride |
| [³ H]spiperone | Pramipexole |
| 7-OH-DPAT | Quinpirole |
| Apomorphine | Ropinirole |
| Chlorpromazine | Rotigotine |

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013).

Introduction

Parkinson's disease (PD) is a chronic, degenerative neurological disease which is characterized by the progressive loss of nigrostriatal dopaminergic pathways giving rise to a range of motor symptoms including rigidity, tremor and bradykinesia. Currently available medications improve signs and symptoms for several years but do not slow the progression of the underlying disease. These medications focus on replacing, or compensating for, the central dopamine deficiency. Levodopa has been the gold standard therapy for the treatment of PD, but it is now clear that the initial control of motor symptoms is confounded after long-term treatment by the development of motor complications (dyskinesias; Jenner, 1995; 2008). Recent advances in medication have focused on the development of novel non-ergoline dopaminergic agonists, as ergoline agonists are associated with rare but severe side effects such as cardiac valve fibrosis (Tan, 2003). These novel dopamine agonists include rotigotine, ropinirole and pramipexole, all of which show high efficacy in reducing motor symptoms with a reduced propensity to induce dyskinesias compared with levodopa especially in younger patients (Clarke and Guttman, 2002; Needham and Worth, 2012). This has led to the use of dopamine agonists as monotherapy in early/mild PD to avoid or delay the need for levodopa (Wood, 2010). Dopamine agonists vary in their pharmacokinetic properties and mode of administration, with many of the drugs requiring repeat daily administration (see Rascol *et al.*, 2007).

Dopamine receptors (see Schwartz *et al.*, 1998; Beaulieu and Gainetdinov, 2011) are divided into two subclasses: the D₁-like which consists of D₁ and D₅ receptors and the D₂-like which includes D₂, D₃ and D₄ receptors. The dopamine D₂ receptor exists as a short and long splice variant (D₂S and D₂L with a 29 amino acid insert in the third intracellular loop), while the D₄ receptor is highly polymorphic in humans with a repeat region in the third cytoplasmic loop. Agonist binding to the D₂-like receptors results in activation of a variety of signalling pathways including inhibition of adenylyl cyclase, which is sensitive to the effects of *Pertussis* toxin, indicating that these responses are mediated by G_{i/o} proteins. All of the non-ergoline agonists currently in clinical use share the property of binding and activating the D₂-like family of dopamine

receptors, although they differ in their relative efficacy at these receptors (Millan *et al.*, 2002; Newman-Tancredi *et al.*, 2002). Thus, pramipexole preferentially stimulates dopamine D₃ receptors compared with D₂ receptors, whereas ropinirole stimulates both dopamine D₂ and D₃ receptors. Although the D₄ receptor is also in the D₂-like family, little is known concerning its role in relation to basal ganglia circuits and motor function. The D₄ receptor is present within the prefrontal cortex, amygdala, hippocampus, thalamus, caudate putamen and cerebellum with lower levels in the mid-brain (see Lauzon and Laviolette, 2010). Selective antagonists (see Beaulieu and Gainetdinov, 2011) exist which preferentially block the D₂, D₃ and D₄ receptors. The dopamine D₁ and D₅ receptors activate the G_{α_{s/olf}} family of G-proteins to stimulate cAMP production. They are found exclusively postsynaptically, they lack introns in their coding regions and are 80% homologous in their transmembrane domains. The D₁ subtype is the predominant form and is localized in the primary dopaminergic projection areas such as striatum, nucleus accumbens and cortical areas whereas the D₅ subtype is expressed in cortical, subcortical and limbic areas (Kahn *et al.*, 2000). There is currently no selective agent, either agonist or antagonist, which discriminates between D₁ and D₅ receptors.

Rotigotine ([-]-2-(*N*-propyl-*N*-2-thienylethylamino)-5-hydroxytetralin) was launched as a non-ergoline dopamine receptor agonist for the treatment of idiopathic PD (Baldwin and Keating, 2007) and for the treatment of restless legs syndrome (Baldwin and Keating, 2008; Oertel *et al.*, 2011). *In vitro* receptor binding studies (Scheller *et al.*, 2009) have shown that rotigotine has a 10-fold selectivity for D₃ (pKi 9.2) receptors compared with D₂, D₄ and D₅ (pKi 8.5–8.0) and a 100-fold selectivity compared with D₁ receptors (pKi 7.2). In functional studies, rotigotine behaved as full agonist at all dopamine receptors but notably the potency for stimulation of D₁ receptors was similar to that for D₂ and D₃ receptors (pEC₅₀ respectively: 9.0, 9.4–8.6, 9.7). Differences between binding affinities and functional potency can be the result of many factors such as receptor reserve and different affinity states. For other GPCRs where agonist and antagonist radioligands exist, marked differences in agonist and antagonist potency are often seen. For example, at the muscarinic M₁ receptor, unlike antagonist ligands, agonist ligands displayed

marked differences in their potency to inhibit the binding of the agonist radioligand [^3H]oxotremorine-M and the antagonist radioligand [^3H]N-methyl scopolamine (Freedman *et al.*, 1988). These differences reflect the presence of high- and low-affinity agonist states of the GPCR (DeLean *et al.*, 1980) and have been demonstrated for a variety of GPCRs (Watson *et al.*, 2000).

We have studied further the pharmacological interaction of rotigotine with all the dopamine receptors and compared this with other dopamine agonists used in the treatment of PD. We have performed these using two different approaches. First, we used radioligand binding comparing agonist and antagonist radioligands. We compared the receptor binding profile of the agonists used in the treatment of PD and of pharmacological standards on human recombinant dopamine D₁, D₂, D₃, D₄ and D₅ receptors using both agonist [^3H]rotigotine (Van der Weide *et al.*, 1987) and [^3H]antagonist radioligands to label these receptors. Second, we have studied the functional interaction of these compounds with the D₁, D₂, D₃, D₄ and D₅ receptors. Functional studies in D₁, D₂ and D₃ receptors were performed using the technique of cellular dielectric spectroscopy (CDS, so-called 'label-free') performed with the Molecular Devices (Wokingham, UK). CDS measures the changes in impedance of a cell layer that occur in response to receptor stimulation which reflects a morphological change in the cells and, as such, is independent of the signalling pathway utilized (Peters *et al.*, 2010). Changes (both positive and negative) in cellular impedance are measured in real time in live cells and can be used to quantify various responses including full/partial agonism, antagonism and allosteric modulation (Rocheville and Jerman, 2009). The response measured represents an integrated response probably reflecting multiple cellular events downstream of the initial receptor event; for example, dopamine D₁ and D₂ receptors couple to different G-proteins. The advantage of using CDS is that we could study receptor activation at the three dopamine receptors using a single methodology. However, this method requires the use of adherent cells which attach to the electrode plate. For the human dopamine D₄ receptor, the receptor was expressed in CHO cells grown in suspension and attempts to attach the cells to the plates using various coating did not give reproducible results. We therefore used agonist-induced G-protein activation as a functional measure at the D₄ receptor. Our efforts to generate a human D₅ receptor expressing cell line failed to yield sufficient expression for radioligand binding studies. The human D₅ receptor cell line was therefore obtained from a commercial source which was coupled to measure changes in intracellular calcium.

Methods

Cell culture

All cells were cultured at 37°C in a humidified atmosphere of 5% CO₂. Cells were grown in DMEM-F12 + GlutaMAX™-I medium (GIBCO®, Invitrogen, Merelbeke, Belgium) containing 10% FBS (BioWhittaker®, Lonza, Verviers, Belgium), 400 µg·mL⁻¹ Geneticin (GIBCO), 100 IU·mL⁻¹ penicillin and 100 IU·mL⁻¹ streptomycin (Pen-Strep solution, BioWhittaker). LMtk (Ltk-) mouse fibroblast cells expressing the dopa-

mine D₁ receptor (BioSignal Inc, Montreal, Canada, now Perkin Elmer) were used as they have been shown to couple efficiently and give robust functional responses (Watts *et al.*, 1995). CHO cells expressing the human dopamine D₂ (long isoform which contains an additional 29 amino acids), D₃ and D₄ (long form with seven repeat polymorphism of exon III) receptors were developed in house. Cells stably expressing the human dopamine D₅ receptor were obtained from Millipore (Merck-Millipore S.A/N.V., Overijse, Belgium) and cultured according to the manufacturer's instructions.

Membrane preparation

Adherent cells were cultured in 176 cm² Petri dishes until confluent and the medium was removed. The cells were washed with 30 mL PBS at 25°C and detached by incubation with 30 mL 1 mM EDTA solution in PBS (pH 7.4) for 7 min at 37°C. After centrifugation (1500× *g* for 10 min at 4°C), the pellet was resuspended in 3 mL buffer [15 mM Tris-HCl buffer (pH 7.4) containing 1 mM EGTA, 0.3 mM EDTA, 2 mM MgCl₂ with one tablet of Complete® Mini EDTA free (Roche, Vilvoorde, Belgium) per 20 mL buffer] per flask. The cells were homogenized (Potter) and the homogenates were frozen in liquid nitrogen and defrosted in a 25°C water bath. This step was repeated once more to complete the cell disruption. After equilibration at 25°C, DNase (final concentration 10 IU·mL⁻¹) was added to the membrane suspension and incubated for 10 min at 25°C followed by centrifugation (40 000× *g* for 25 min at 4°C). The pellet was resuspended in Tris-sucrose buffer (20 mM Tris-HCl buffer pH 7.4 containing 250 mM sucrose). The membrane preparation was frozen in aliquots in liquid nitrogen before storage at -140°C. Suspension cells were centrifuged and treated as described earlier.

Radioligand binding assays

Binding assays were performed in 96-well polypropylene tubes in a final volume of 2 mL for D₁ and D₄ membranes and 1 mL for D₂, D₃ and D₅ membranes containing: 50 µL radioligand, 10 µL drug/buffer/non-specific binding, buffer (final concentration 50 mM Tris-HCl pH 7.4, MgCl₂ 2 mM) and membranes (5 µg protein for D₂ and D₃ and 25 µg protein for D₁ and D₅). Following 120 min of incubation at 25°C, bound radioligand was determined by rapid vacuum filtration through A/C glass fibre filters (Pall Corporation, Zaventem, Belgium) presoaked in 0.1% polyethylenimine. The filters were washed four times with 2 mL ice-cold washing buffer (Tris-HCl 50 mM, pH 7.4 at 4°C) and retained radioactivity was determined by liquid scintillation counting.

In kinetic studies, association and dissociation [induced by an excess (10 µM) of chlorpromazine for D₂, D₃ and D₄ receptors and apomorphine for D₁ and D₅ receptors] were followed at different times up to 180 min.

For saturation studies, the concentration of radioligand used was typically 0.02–5 nM. For competition and kinetic studies, [^3H]rotigotine was used at 0.8 nM for dopamine D₁ and 0.2 nM for dopamine D₂, D₃, D₄ and D₅ receptors. For antagonist radioligands in competition studies, [^3H]SCH23390 was used at 0.03 nM for D₁ and D₅ receptors, [^3H]raclopride at 0.65 nM for D₂ receptors and [^3H]spiperone at 0.3 nM for D₃ receptors and 0.05 nM for D₄ receptors. These conditions were selected to give a robust signal window

in the absence of ligand depletion (which was observed for [³H]spiperone in the CHO D₂ cells). Competition curves were performed using 10 concentrations (half-log dilutions) in triplicate.

CDS

CDS measurements were performed with the Cellkey (MDS Sciex) at 37°C. Cells were seeded into the wells of a 96-well microplate in 200 µL medium and incubated overnight (37°C; 5% CO₂). Growth medium was then exchanged to 135 µL incubation buffer [Hank's balanced salt solution (HBSS) containing 20 mM HEPES, pH 7.4]. Plates were placed onto the Cellkey system and baseline measurements were taken for 5 min. The test agonist (15 µL) at varying concentrations (ranging from 0.1 pM to 10 µM) was added and impedance measurements were collected for 30 min.

[³⁵S]GTPγS binding

Membranes (CHO hD₄, 20 µg per assay) were incubated with TRIS-MgCl₂, drug/H₂O/agonist, containing final concentrations: NaCl 50 mM; MgCl₂ 3 mM; GDP 1 µM; saponin 10 µg·mL⁻¹ in 200 µL for 15 min at 25°C. Then, 20 µL of [³⁵S]GTPγS (0.15–0.20 nM in 0.01N HCl) was added to each well and incubated for 60 min at 25°C. The plates were filtered, dried and retained radioactivity was determined.

Intracellular calcium

Changes in intracellular calcium were followed in cells stably expressing the human dopamine D₅ receptor and G-protein Gα₁₅ (see Smart and Wood, 2001). Cells were pre-incubated for 60 min with 4 µM Fluo-4 AM (Molecular Probes F-14202; Life Technologies, Gent, Belgium) with pluronic acid in the presence of probenecid (0.8 mM final). Cells (20 000 per well) were washed and incubated in HBSS with 20 mM HEPES (pH 7.4), basal readings taken, drugs added and changes in intracellular calcium were determined using a FlexStation (Molecular Devices, St. Grégoire Cédex, France).

Data analysis

Data were analysed using Excel and PRISM (GraphPad Software, La Jolla, CA, USA). Association and dissociation data were fitted to one-phase and two-phase models and the best fit was determined using the extra sum-of-squares F-test (PRISM). Equilibrium saturation curves were analysed in Microsoft Office Excel 2003 using one-site or two-site analysis, depending on the outcome of the sum-of-squares F-test performed with PRISM. Competition curves were fitted to the three-parameter logistic equation (slope fixed to unity as the slope was not significantly different from 1) and pIC₅₀ corrected to pK_i according to Cheng and Prusoff (1973). In functional studies, EC₅₀ values were determined by non-linear regression analysis of the curves using the log(agonist) versus response model in PRISM (slope = 1 as it was not significantly different from unity).

Materials

All cell culture reagents were obtained from Invitrogen unless otherwise stated.

Radioligands used were: [³H]rotigotine (54 Ci·mmol⁻¹, Tritec AG, Teufen, Switzerland); [³H]SCH23390 (88 Ci·mmol⁻¹,

GE Healthcare, Diegem, Belgium), [³H]raclopride (83 Ci·mmol⁻¹, Perkin Elmer, Zaventem, Belgium) and [³H]spiperone (15 Ci·mmol⁻¹, Perkin Elmer). Rotigotine was synthesized at UCB (Braine-l'Alleud, Belgium), reagents and standards were obtained from Sigma-RBI (Diegem, Belgium).

Results

Kinetics of [³H]rotigotine binding

The binding of [³H]rotigotine to all dopamine receptor subtypes was reversible and showed time dependence. Although dissociation from the D₂ and D₃ receptors was slow, a partial inhibition model did not better describe the data than a complete inhibition model; therefore, data were analysed assuming complete reversibility would be reached. The association of [³H]rotigotine with the dopamine D₂, D₄ and D₅ receptors was best described by a two-phase model, whereas the dopamine D₁ and D₃ receptor association data were best described by a one-phase model (Figure 1). The dissociation data at all receptors were best described by a one-phase model. Binding kinetic constants are shown in Table 1.

Comparison of saturation binding of agonist and antagonist binding at dopamine D₁, D₂, D₃, D₄ and D₅ receptors

Equilibrium saturation assays were performed in triplicate with [³H]rotigotine and with the radiolabelled antagonist [³H]SCH23390 for D₁ and D₅, [³H]raclopride for D₂ and [³H]spiperone for D₃ and D₄ receptors (Table 2 and Figure 2). Analysis of the saturation binding of [³H]rotigotine to dopamine D₁ and D₂ receptors was best described by a two-site model and by a one-site model for the D₃, D₄ and D₅ receptors. For the two-site analysis, the B_{max} of the low-affinity site was poorly defined because of the low signal at high radiolabel concentrations but the fit was well described assuming a similar B_{max} value to that seen with the antagonist radioligand. At the dopamine D₁ and D₅ receptors, the number of sites (B_{max}) identified by [³H]rotigotine was markedly lower than that labelled by [³H]SCH23390. At the D₂, D₃ and D₄ receptors, the number of sites labelled by [³H]rotigotine was similar to that labelled by [³H]raclopride and [³H]spiperone respectively. At the dopamine D₁ receptor, the presence of 5'-guanylyl imidodiphosphate (GppNHp; 10 µM) attenuated the high-affinity [³H]rotigotine binding site whereas GppNHp had no significant effect on [³H]rotigotine binding to the D₂ and D₃ receptors.

Competition profile at human dopamine D₁, D₂, D₃, D₄ and D₅ receptors

To compare the pharmacological profile of the site labelled by the agonist radioligand [³H]rotigotine with that labelled by antagonist radioligands, competition binding assays were performed. The assays were carried out using both [³H]rotigotine as the agonist radioligand and using [³H]SCH23390, [³H]raclopride and [³H]spiperone as the antagonist radioligand for D₁ and D₅, D₂, D₃ and D₄ receptors respectively (Table 3). Rotigotine itself displaced [³H]rotigotine binding at the human dopamine receptors with pK_i values in agree-

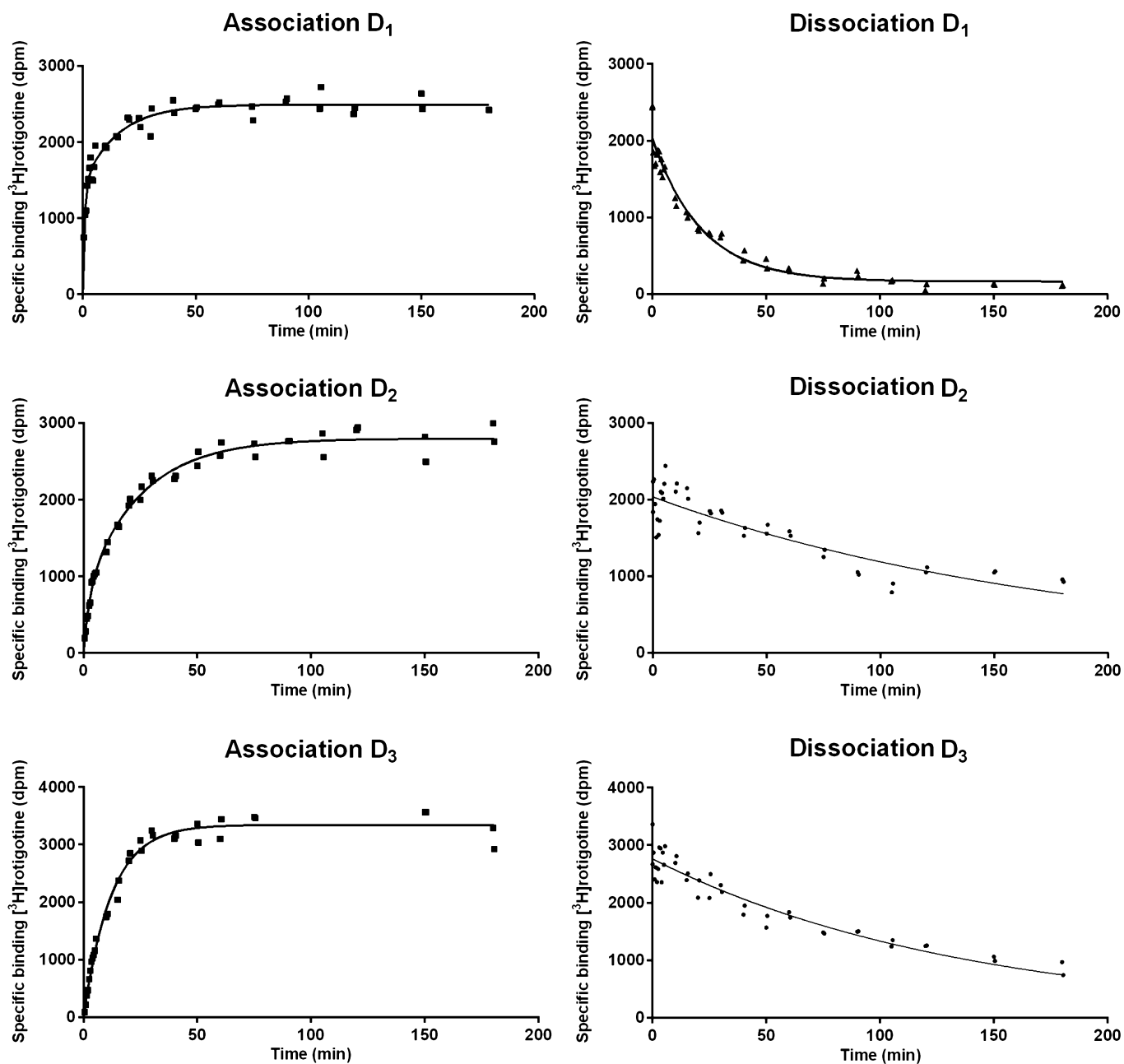


Figure 1

Association and dissociation curves for [³H]rotigotine binding to human dopamine D₁, D₂ and D₃ receptors. Data are representative of three independent experiments. The curves are the best fit as described with calculated constants shown in Table 1.

ment with the measured pK_D. At the D₁ and D₂ receptors, the observed pK_i was consistent with the high-affinity site observed in saturation experiments (pK_D 9.7 and 10.0 respectively).

For the antagonist compounds, SB277011A (Reavill *et al.*, 2000) displayed some selectivity for the dopamine D₃ receptor; SCH23390 displayed marked selectivity for the dopamine D₁ and D₅ receptors and raclopride, spiperone and haloperidol exhibited high affinity for the dopamine D₂, D₃ and D₄ receptors. There was a marked similarity in binding affinities for all

of these antagonists at each dopamine receptor labelled with either [³H]rotigotine or the respective antagonist radioligand.

For the agonist compounds, some marked differences were seen. The agonists pramipexole, ropinirole, (-)-3-(3-hydroxyphenyl)-N-n-piperidine ((-)-3-PPP) and quinpirole lacked affinity for the D₁ and D₅ receptors. All of the other agonists displayed a higher affinity for the D₁ receptor labelled with [³H]rotigotine compared with the antagonist ligand, [³H]SCH23390. At the dopamine D₂ receptor, all of the agonists inhibited [³H]rotigotine binding with a higher affin-

Table 1Radioligand binding kinetic constants of [³H]rotigotine binding to human dopamine D₁, D₂, D₃ and D₄ receptors

| Kinetic parameters | Receptor | | | | |
|--|----------------|----------------|----------------|----------------|----------------|
| | D ₁ | D ₂ | D ₃ | D ₄ | D ₅ |
| Dissociation | | | | | |
| k _{off} (min ⁻¹) | 0.08 ± 0.01 | 0.007 ± 0.002 | 0.008 ± 0.001 | 0.03 ± 0.01 | 0.16 ± 0.01 |
| t _{1/2} (min) | 8.6 ± 0.2 | 110 ± 30 | 87 ± 7 | 24 ± 3.5 | 4.5 ± 0.4 |
| Association | | | | | |
| k _{obs} fast (min ⁻¹) | 1.2 ± 0.2 | 0.42 ± 0.09 | 0.11 ± 0.02 | 0.26 ± 0.04 | 0.25 ± 0.08 |
| k _{obs} slow (min ⁻¹) | 0.06 ± 0.03 | 0.04 ± 0.01 | – | 0.035 ± 0.006 | – |
| %fast | 45 ± 2 | 70 ± 6 | – | 84 ± 9 | – |
| t _{1/2} fast (min) | 0.56 ± 0.07 | 1.7 ± 0.3 | 6.62 ± 1.6 | 2.7 ± 0.4 | 1.5 ± 0.7 |
| t _{1/2} slow (min) | 12 ± 5 | 19 ± 4 | – | 25 ± 4 | – |

Results are mean ± SD from three separate experiments. Kinetic constants were determined as described. k_{off} is the dissociation rate constant and k_{obs} = k_{on}L + k_{off} where k_{on} is the association rate constant and L is the concentration of radioligand. t_{1/2} is the half time for dissociation or association.

Table 2Equilibrium binding constants for [³H]rotigotine and [³H]antagonist radioligands at human recombinant dopamine receptors

| Receptor | Radioligand | K _D (nM) | | B _{max} (fmol·mg ⁻¹ protein) | |
|----------------|---------------------------------------|---------------------|-------------------|--|-------------------|
| | | High affinity site | Low affinity site | High affinity site | Low affinity site |
| D ₁ | [³ H]rotigotine | 0.19 ± 0.13 | 6.80 ± 3.23 | 440 ± 420 | 2 970 ± 400 |
| | [³ H]rotigotine +Gpp(NH)p | – | 10.0 ± 5.5 | – | 2 700 ± 1 300 |
| | [³ H]SCH23390 | 0.69 ± 0.23 | – | 29 700 ± 5 500 | – |
| D ₂ | [³ H]rotigotine | 0.09 ± 0.02 | 4.57 ± 4.4 | 6 390 ± 3 600 | 20 530 ± 4 600 |
| | [³ H]rotigotine +Gpp(NH)p | 0.20 ± 0.14 | 4.08 ± 3.9 | 4 120 ± 2 300 | 23 300 ± 9 300 |
| | [³ H]raclopride | 0.68 ± 0.07 | – | 17 600 ± 2 600 | – |
| D ₃ | [³ H]rotigotine | 0.21 ± 0.06 | – | 22 700 ± 5 600 | – |
| | [³ H]rotigotine +Gpp(NH)p | 0.20 ± 0.04 | – | 26 540 ± 1 545 | – |
| | [³ H]spiperone | 0.12 ± 0.03 | – | 18 100 ± 950 | – |
| D ₃ | [³ H]rotigotine | 0.83 ± 0.06 | – | 1 550 ± 640 | – |
| | [³ H]spiperone | 0.055 ± 0.01 | – | 1 440 ± 670 | – |
| D ₄ | [³ H]rotigotine | 6.50 ± 1.85 | – | 1 300 ± 290 | – |
| | [³ H]SCH23390 | 1.08 ± 0.44 | – | 8 200 ± 1 200 | – |

Data shown are the mean ± SD calculated parameters from three to four saturation experiments. Parameters were calculated in PRISM using one-site or two-site analysis, depending on the outcome of the sum-of-squares F-test.

ity than required to inhibit binding of the antagonist [³H]raclopride. This difference in inhibitory affinity was greater at the dopamine D₁ receptor. At the dopamine D₃ receptor, there was no difference in inhibitory affinity for these agonists using either agonist ([³H]rotigotine) or antagonist ([³H]spiperone) radioligands. The agonists apomorphine, dopamine and rotigotine all displayed similar affinities for the [³H]rotigotine binding site on the dopamine D₁, D₂ and D₃ receptors. The agonists lisuride, pramipexole, ropinirole, (-)-3-PPP, quinpirole and 7-OH-DPAT all showed selectivity for the dopamine D₂ and D₃ receptor compared with the D₁ receptor. Further,

pramipexole and 7-OH-DPAT showed some selectivity for the dopamine D₃ receptor which was more apparent when using [³H]antagonist ligands than [³H]rotigotine.

At the dopamine D₄ receptor, most of the agonists tested displayed a lower affinity for this receptor (on both [³H]rotigotine and [³H]spiperone/antagonist binding) than for the D₂ and D₃ receptors. There was also a tendency for the potency to be higher on [³H]rotigotine binding than on [³H]spiperone which was most marked with dopamine. Dopamine itself exhibited similar affinity for the D₂, D₃ and D₄ receptors.

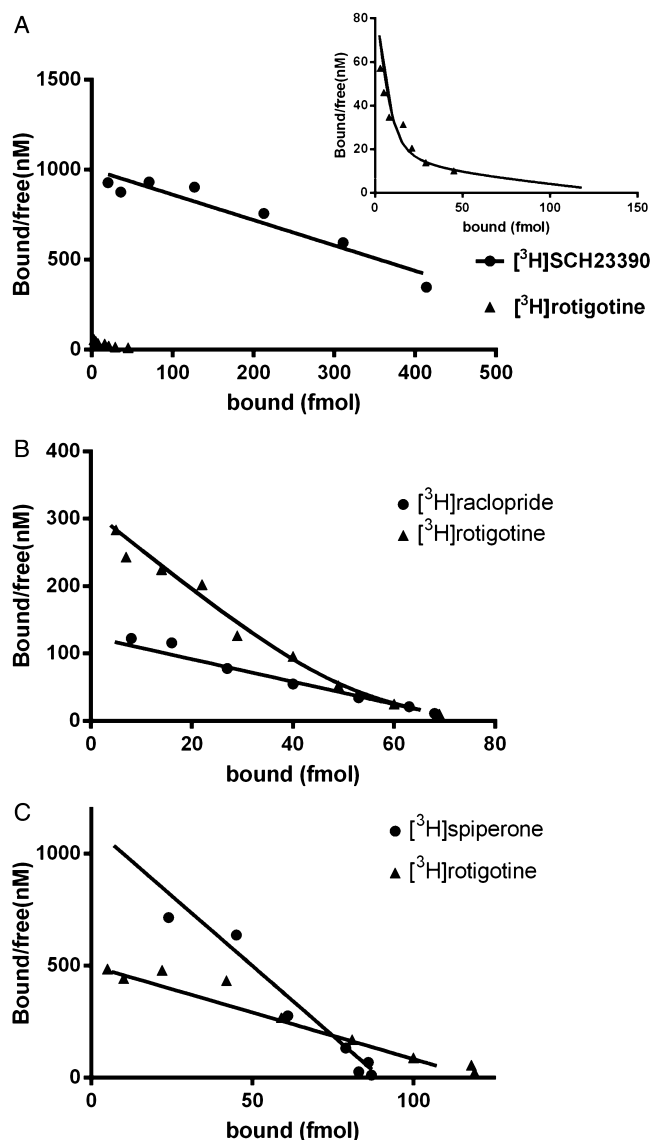


Figure 2

Representative Scatchard plots from radioligand saturation binding studies. (A) Shows the specific binding of [³H]SCH23390 and [³H]rotigotine to the human dopamine D₁ receptor expressed in LMtk cells with inset showing enlargement to show two-site fit for [³H]rotigotine. (B) Shows the specific binding of [³H]raclopride and [³H]rotigotine to the human dopamine D₂ receptor expressed in CHO cells. (C) Shows the specific binding of [³H]spiperone and [³H]rotigotine to the human dopamine D₃ receptor expressed in CHO cells.

For the dopamine D₅ receptor, although the pharmacological profile was similar to that seen at the D₁ receptor, the difference in inhibitory potency of agonist ligands at the agonist ([³H]rotigotine) and antagonist ([³H]SCH23390) radioligand binding was not as marked as that seen at the D₁ receptor.

Functional profile at human dopamine D₁, D₂, D₃, D₄ and D₅ receptors

The optimal cell density was determined using 10 μM dopamine and 10 μM ATP: 50 000 cells per well was chosen for

LMtk D₁, 30 000 cells per well for CHO D₂ and 35 000 cells per well for CHO D₃ cells to perform the functional assays.

In LMtk D₁ cells, dopamine produced a concentration-dependent decrease in cellular impedance [maximal response 25–30 dZiec (Ohms)]. In D₂ CHO and D₃ CHO cells, dopamine produced a concentration-dependent increase in cellular impedance with a maximal response around 80 dZiec (Ohms) for D₂ and 40 dZiec (Ohms) for D₃.

Almost all of the compounds tested appeared as full agonists at the dopamine D₁, D₂ and D₃ receptors in that they all produced maximal responses similar to that of the endogenous agonist dopamine (data shown for D₁ receptor Figure 3). The exception was (-)-3-PPP which gave a submaximal response at the dopamine D₂ receptor (88% compared with dopamine 100%, *P* < 0.05 Student's paired *t*-test). There was a good correlation between functional potency of the agonists examined (Table 4) and their binding affinity versus [³H]rotigotine (Table 5) at the three dopamine receptors (Figure 4). At the dopamine D₂ receptor, there is a good correlation between functional potency and binding affinity when using [³H]rotigotine as an agonist radioligand and when using [³H]spiperone as an antagonist radioligand (Figure 4), but there is a 100-fold lower binding affinity. At the dopamine D₃ receptor, although there was a good correlation, the functional potency of compounds tended to be lower than their binding affinity at the D₃ receptor and this was most notable for rotigotine, lisuride and pramipexole. Rotigotine, lisuride, dopamine and apomorphine displayed agonist properties at all three dopamine receptors, whereas pramipexole, ropinirole, (-)-3-PPP and quinpirole only displayed agonist activity at dopamine D₂ and D₃ receptors.

At the dopamine D₄ receptor, most compounds (except apomorphine) displayed a lower potency compared with the D₂ and D₃ receptors. Further, all of the agonists appeared as partial agonists compared with dopamine, with efficacy of 60–80% with respect to dopamine maximal response. Similarly, at the D₅ receptor, those agonists tested displayed a lower potency than that seen at the dopamine D₁ receptor and they appeared as partial agonists.

Discussion and conclusions

In preclinical studies, rotigotine has been shown to act as a dopamine D₂ receptor agonist (Van der Weide *et al.*, 1987; Tan, 2003; Rascol *et al.*, 2007). In radioligand binding studies (Scheller *et al.*, 2009), rotigotine exhibited the highest affinity for the dopamine D₃ receptor (K_i 0.71 nM), high affinity for D₂, D₅ and D₄ receptors (K_i 4–15 nM) and low affinity for the dopamine D₁ receptor (K_i 83 nM). However, in functional studies in the same paper, it was found that rotigotine acts as an agonist at all dopamine receptors with a moderate selectivity for the dopamine D₂ receptor-like family. In the present study, we show that rotigotine is a high-affinity agonist at several dopamine receptors, notably D₁, D₂ and D₃.

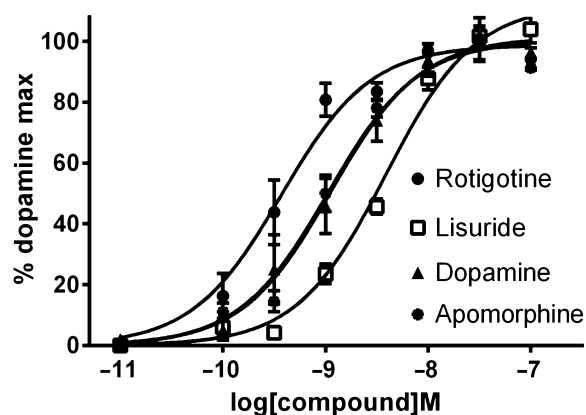
Using [³H]rotigotine as an agonist radioligand, we show that it can be used to label all the dopamine receptors and that it displays a high affinity for dopamine D₁, D₂ and D₃ receptors with a lower affinity for dopamine D₄ and D₅ receptors. Further, there is evidence that [³H]rotigotine labels an agonist state at some of these receptors. In kinetic studies,

Table 3

Affinity of selected compounds for human recombinant dopamine D₂, D₃ and D₄ receptors identified using both [³H]rotigotine and [³H]standard antagonist in radioligand binding studies

| Compounds | pK _i D ₂ | | pK _i D ₃ | | pK _i D ₄ | |
|--------------------|--------------------------------|-----------------------|--------------------------------|----------------------|--------------------------------|-----------------------|
| | [³ H]Rotig | [³ H]Spip | [³ H]Rotig | [³ H]Rac | [³ H]Rotig | [³ H]Spip |
| Agonists | | | | | | |
| Rotigotine | 10.2 ± 0.2 | 8.7 ± 0.1 | 9.9 ± 0.1 | 10.0 ± 0.2 | 8.5 ± 0.1 | 7.7 ± 0.5 |
| Lisuride | 9.3 ± 0.4 | 8.3 ± 0.2 | 8.5 ± 0.1 | 8.9 ± 0.1 | 6.8 ± 0.1 | 6.8 ± 0.1 |
| Pramipexole | 8.6 ± 0.1 | 6.7 ± 0.1 | 9.4 ± 0.1 | 9.6 ± 0.3 | 7.9 ± 0.1 | 7.2 ± 0.1 |
| Ropinirole | 7.8 ± 0.1 | 6.4 ± 0.1 | 8.2 ± 0.1 | 8.0 ± 0.1 | 6.9 ± 0.1 | 6.6 ± 0.1 |
| Dopamine | 8.5 ± 0.1 | 6.7 ± 0.2 | 8.7 ± 0.2 | 8.5 ± 0.1 | 8.2 ± 0.2 | 7.1 ± 0.1 |
| (-)3-PPP | 7.2 ± 0.2 | 5.5 ± 0.2 | 7.3 ± 0.1 | 7.0 ± 0.1 | 7.0 ± 0.1 | 6.2 ± 0.2 |
| Quinpirole | 8.2 ± 0.2 | 6.5 ± 0.1 | 8.7 ± 0.1 | 8.7 ± 0.1 | 8.1 ± 0.1 | 7.6 ± 0.1 |
| Apomorphine | 9.3 ± 0.1 | 8.0 ± 0.1 | 8.3 ± 0.1 | 8.4 ± 0.2 | 8.9 ± 0.1 | 8.4 ± 0.2 |
| 7-OH-DPAT | 8.4 ± 0.3 | 7.1 ± 0.2 | 9.1 ± 0.1 | 9.1 ± 0.1 | – | – |
| Antagonists | | | | | | |
| SCH23390 | 7.2 ± 0.1 | 6.9 ± 0.4 | 6.3 ± 0.1 | 6.2 ± 0.1 | 6.7 ± 0.5 | 6.6 ± 0.1 |
| Raclopride | 8.7 ± 0.1 | 9.1 ± 0.3 | 7.0 ± 0.1 | 7.1 ± 0.1 | 5.7 ± 0.5 | 5.8 ± 0.3 |
| Haloperidol | 10.5 ± 0.1 | 9.9 ± 0.5 | 8.4 ± 0.2 | 8.7 ± 0.1 | – | – |
| Spiperone | 10.3 ± 0.1 | 10.4 ± 0.2 | 9.6 ± 0.2 | 9.9 ± 0.1 | 9.8 ± 0.2 | 10.1 ± 0.2 |
| SB277011A | 6.3 ± 0.2 | 6.2 ± 0.1 | 7.8 ± 0.1 | 7.7 ± 0.1 | 5.4 ± 0.2 | 5.6 ± 0.2 |

Data are mean ± SD from three separate experiments. Shown are the inhibitory affinity constants (pK_i) to inhibit the binding of [³H]rotigotine (Rotig), [³H]spiperone (Spip) and [³H]raclopride (Rac).

**Figure 3**

The effect of rotigotine and other dopamine agonists on the human dopamine D₁ receptor in functional studies on LMtk cells. Results are mean changes in cellular dielectric spectroscopy (with SEM) expressed as a % of the maximum fitted response to the dopamine concentration-response curve from three separate determinations.

there was evidence for the presence of two states of the D₁, D₂, D₄ and D₅ receptors whereas only one state was evident for the D₃ receptor. Interestingly, this was seen in association studies but was not evident in dissociation studies. There was also evidence in saturation studies where two affinity states were observed for the D₁ and D₂ receptors. This profile of

multiple affinity states, sensitivity to guanyl nucleotides and reduced binding capacity compared with antagonist radioligands, is consistent with [³H]rotigotine being an agonist at these GPCRs. The lack of this profile at the dopamine D₃ receptor may reflect the poor functional coupling of this receptor to G-proteins and its selective coupling to G_o (Zaworski *et al.*, 1999; Lane *et al.*, 2008). For the dopamine D₂ receptor, where two affinity states for [³H]rotigotine were observed which were insensitive to the effects of GppNHp, these states may represent different conformations of the D₂ receptor bound to different G-proteins present in the cell. The D₂ receptor couples promiscuously to several members of the G_{α_{i/o}} family for which it displays different potencies (Lane *et al.*, 2008) and this may be apparent in cell lines where the D₂ receptor is overexpressed giving rise to apparent multiple binding states. In competition binding studies, the pharmacological profile of [³H]rotigotine binding was again consistent with labelling an agonist state. Thus, in general, agonists, and notably dopamine, were more potent displacers of [³H]rotigotine binding than of the [³H]antagonist binding at all dopamine receptors except the D₃ receptor. This may reflect the poor functional coupling seen with the D₃ receptor as noted earlier.

Further, we show that rotigotine is a high potency agonist at all dopamine receptors, notably D₁, D₂ and D₃. Indeed, rotigotine exhibited subnanomolar potency for the dopamine D₁ and D₂ receptors, low nanomolar potency for the D₃ receptor and high nanomolar potency for the D₄ and D₅ receptors in functional studies. Although this is the first study

Table 4

Functional profile of dopaminergic agonists at human recombinant dopamine receptor subtypes

| Compound | Receptor | | | | | | |
|-------------|-------------------------------------|-------------------------------------|-------------------------------------|-------------------------------------|----------------------------|-------------------------------------|----------------------------|
| | D ₁ pEC ₅₀ | D ₂ pEC ₅₀ | D ₃ pEC ₅₀ | D ₄ pEC ₅₀ | D ₄ Emax (%) | D ₅ pEC ₅₀ | D ₅ Emax (%) |
| Rotigotine | 9.6 ± 0.1 | 10.4 ± 0.1 | 8.2 ± 0.2 | 7.7 ± 0.1 | 62 ± 2 | 7.7 ± 0.2 | 64 ± 5 |
| Lisuride | 8.5 ± 0.1 | 8.4 ± 0.1 | 6.8 ± 0.2 | – | – | – | – |
| Pramipexole | <5 | 9.1 ± 0.2 | 8.3 ± 0.1 | 7.4 ± 0.1 | 62 ± 4 | <5 | – |
| Ropinirole | <5 | 8.5 ± 0.1 | 8.1 ± 0.1 | 6.3 ± 0.1 | 67 ± 6 | <5 | – |
| Dopamine | 9.0 ± 0.4 | 8.6 ± 0.3 | 8.3 ± 0.1 | 7.5 ± 0.1 | 100 | 8.2 ± 0.3 | 100 |
| (-)-3-PPP | <5 | 7.5 ± 0.1 | 7.5 ± 0.1 | 6.4 ± 0.2 | 2 ± 3 | – | – |
| Quinpirole | <5 | 8.9 ± 0.1 | 8.0 ± 0.2 | 7.1 ± 0.5 | 81 ± 9 | <5 | – |
| Apomorphine | 9.1 ± 0.1 | 9.5 ± 0.8 | 8.1 ± 0.1 | 8.3 ± 0.5 | 63 ± 1 | 7.9 ± 0.2 | 80 ± 5 |

Data are mean ± SD from three to four separate experiments.

Table 5Affinity of selected compounds for human recombinant dopamine D₁ and D₅ receptors identified using both [³H]rotigotine and [³H]standard antagonist in radioligand binding studies

| Compounds | pK _i D ₁ | | pK _i D ₅ | |
|-------------|--------------------------------|----------------------|--------------------------------|----------------------|
| | [³ H]Rotig | [³ H]SCH | [³ H]Rotig | [³ H]SCH |
| Agonists | | | | |
| Rotigotine | 9.2 ± 0.1 | 6.6 ± 0.1 | 7.8 ± 0.2 | 7.4 ± 0.1 |
| Lisuride | 7.5 ± 0.1 | 6.0 ± 0.6 | – | – |
| Pramipexole | <5 | <5 | <5 | <5 |
| Ropinirole | <5 | <5 | <5 | <5 |
| Dopamine | 9.0 ± 0.2 | 5.8 ± 0.1 | 8.2 ± 0.1 | 7.5 ± 0.1 |
| (-)-3-PPP | <5 | <5 | <5.5 | <5.5 |
| Quinpirole | <5 | <5 | <5 | <5 |
| Apomorphine | 8.8 ± 0.4 | 6.5 ± 0.1 | 7.9 ± 0.1 | 7.4 ± 0.1 |
| 7-OH-DPAT | 6.7 ± 0.1 | 4.7 ± 0.1 | 6.0 ± 0.1 | 5.2 ± 0.1 |
| Antagonists | | | | |
| SCH23390 | 9.4 ± 0.2 | 9.3 ± 0.2 | 9.2 ± 0.1 | 9.5 ± 0.1 |
| Raclopride | <5 | <5 | <5 | <5 |
| Haloperidol | 8.3 ± 0.2 | 8.1 ± 0.2 | 9.1 ± 0.1 | 9.3 ± 0.6 |
| Spiperone | 6.8 ± 0.1 | 6.6 ± 0.1 | 6.0 ± 0.2 | 6.1 ± 0.1 |
| SB277011A | <5 | <5 | <5 | <5 |

Data are mean ± SD from three separate experiments. Shown are the inhibitory affinity constants (pK_i) to inhibit the binding of [³H]rotigotine (Rotig) and [³H]SCH23390 (SCH).

which has compared functional interactions across all dopamine receptors, the results are in agreement with the limited published studies. The potency values obtained in the present study with ropinirole, pramipexole and dopamine agree with those reported by Coldwell *et al.* (1999) and Perachon *et al.* (1999) who also found these compounds to be full agonists. There was also good agreement on functional potencies at D₃ receptors, with the results of Newman-Tancredi *et al.* (2002)

with the exception of lisuride which was reported to be more potent and a partial agonist. This difference may reflect the slow kinetics of lisuride such that equilibrium may not be reached in functional studies (Coldwell *et al.*, 1999). Recently, the concept of biased signalling or functional selectivity has emerged which may explain some of the differences seen in agonist pharmacology (Urban *et al.*, 2007). However, one mechanism for such functional selectivity involves signalling

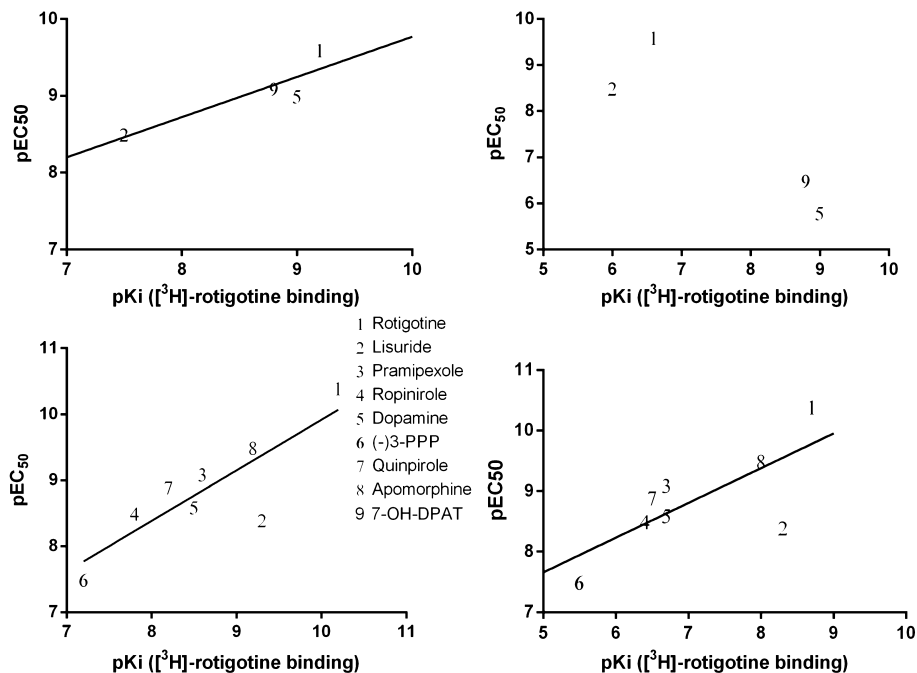


Figure 4

Correlation between functional potency (pEC₅₀) and binding affinity (pKi) at the dopamine D₁ receptor (top panels) and D₂ receptor (bottom panels) using agonist [³H]rotigotine) and antagonist [³H]SCH23390 and [³H]spiperone for D₁ and D₂ respectively).

through multiple G-proteins and, although this has been demonstrated for the dopamine D₂ receptor (Beaulieu *et al.*, 2005), the D₃ receptor has been shown to selectively couple to G_α_{o1} (Lane *et al.*, 2008). It would be interesting to study the effects of these agonists on G-protein activation (e.g. [³⁵S]GTPγS binding) and on other signalling pathways, such as cAMP, ERK, Akt. Potency and efficacy generally tended to be lower at the D₄ receptor compared with the closely related D₂ and D₃ receptors, such that agonists display partial agonist properties consistent with other studies on the D₄ receptor (Newman-Tancredi *et al.*, 2002; Heusler *et al.*, 2009). Similarly, functional potency at the D₃ receptor tended to be lower than that seen at the D₁ receptor. As discussed earlier, functional potency and efficacy can be affected by levels of receptor expression/receptor reserve and by stimulus-response coupling. However, the lower potency at the D₄ and D₅ receptors for the agonists examined was also seen in radioligand binding studies suggesting that this may be a function of the receptor.

On the basis of radioligand binding and functional studies, there therefore appears to be two classes of dopamine agonists. First, apomorphine and rotigotine which display a high affinity and potency for dopamine D₁, D₂ and D₃ receptors and a lower affinity and potency for dopamine D₄ and D₅ receptors. Second, ropinirole, pramipexole and lisuride which display high affinity and potency for the dopamine D₂ and D₃ receptors, lower affinity and potency for dopamine D₄ receptors and with negligible activity at the dopamine D₁ and D₅ receptors. This may have clinical implications in the treatment of PD as, despite the recent advances with the use of dopamine agonists, levodopa remains the gold standard of symptomatic efficacy in treating the motor deficits in PD

(Poewe, 2009). It is generally accepted that levodopa (after conversion to dopamine) acts via stimulation of all dopamine receptors. Because there are similar numbers of dopamine D₁ and dopamine D₂ receptors in the striatum, simultaneous stimulation of both receptor types may be one of the explanations for the greater efficacy of levodopa. In support of this, intermittent s.c. injections of apomorphine, which is a full agonist at dopamine D₁ receptors (*inter alia*; Tiberi and Caron, 1994) and which shows high efficacy at dopamine D₂ receptors (*inter alia*; Newman-Tancredi *et al.*, 2002), have been shown to produce anti-Parkinsonian efficacy similar to that of levodopa (Poewe and Wenning, 2000). Unfortunately, the use of apomorphine in the clinic is limited by its high first-pass metabolism and its side effects such as emesis (Poewe and Wenning, 2000). It was therefore proposed that a synergistic activation of both dopamine D₁ and D₂ receptors may be important for full reversal of Parkinsonian motor deficits (Robertson, 1992; Vermeulen *et al.*, 1994; 1999). The dopamine D₄ receptor has been suggested to play a role in emotional processing and plasticity and in novelty/impulsive behavioural phenomena (see Lauzon and Laviolette, 2010). As such, it has been suggested that the D₄ receptor may play a role in attention deficit hyperactivity disorder and in the mechanism of action of drugs used in the treatment of such disorders. Impulse-control disorders are a common side effect associated with dopamine agonists (Perez-Lloret and Rascol, 2010). They are seen in 5 to 15% of patients and may affect patients severely, as in the case of pathological gambling). It is tempting to speculate that an action at the D₄ receptor may contribute to these adverse effects. Little is known regarding the role of the dopamine D₅ receptor in the mechanism of action of the dopamine agonists in PD. There are no selective

pharmacological agents which discriminate between D₁ and D₅ receptors. D₅ receptors exhibit much lower concentrations than D₁ receptors and while D₁ receptors are expressed widely throughout the brain, D₅ receptors are restricted in expression patterns, particularly in cortical and limbic regions (see Homes *et al.*, 2001). Evidence from D₅ receptor knockout mice suggest that the D₅ receptor does not play a major role in many dopamine-mediated behaviours, but may contribute to exploratory locomotion, startle and pre-pulse inhibition. As well as a role in locomotor activity, the D₁ receptor plays a role in memory and learning so an agonist action at this receptor may be beneficial in elderly patients where cognitive impairment is often seen.

In conclusion, rotigotine acts as a high-affinity dopamine receptor agonist at D₁, D₂, D₃ and, to a lesser extent, D₄ and D₅ receptor subtypes and, as such, is differentiated from compounds such as ropinirole and pramipexole which act mainly as dopamine D₂, D₃ and, to a lesser extent, D₄ receptor agonists. Indeed, the profile of rotigotine more closely resembles that of apomorphine. The present studies were performed *in vitro* using recombinant expression systems. It will therefore be of interest to study these effects in native tissue systems. It also remains to determine whether this D₁ receptor activation by rotigotine contributes to its *in vivo* profile and whether this translates to its clinical profile in the treatment of PD.

Acknowledgements

We thank Mr David Urbain, Mme Murielle Martini and Mr Michel Famelart for excellent technical assistance and support in this study.

Author contributions

M. W, M. G. and D. S. conceived and designed the experiments. V. D. performed the experiments. M. W. and M. G. wrote the paper.

Conflict of interest

Authors declare that they have not any conflict of interest.

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