

In Vitro and In Vivo Identification of Novel Positive Allosteric Modulators of the Human Dopamine D2 and D3 Receptor[§]

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ABSTRACT

Agonists at dopamine D2 and D3 receptors are important therapeutic agents in the treatment of Parkinson's disease. Compared with the use of agonists, allosteric potentiators offer potential advantages such as temporal, regional, and phasic potentiation of natural signaling, and that of receptor subtype selectivity. We report the identification of a stereoselective interaction of a benzothiazol racemic compound that acts as a positive allosteric modulator (PAM) of the rat and human dopamine D2 and D3 receptors. The R isomer did not directly stimulate the dopamine D2 receptor but potentiated the effects of dopamine. In contrast the S isomer attenuated the effects of the PAM and the effects of dopamine. In radioligand binding studies, these compounds do not compete for binding of orthosteric ligands, but indeed the R isomer increased

the number of high-affinity sites for [³H]-dopamine without affecting K_d . We went on to identify a more potent PAM for use in native receptor systems. This compound potentiated the effects of D2/D3 signaling in vitro in electrophysiologic studies on dissociated striatal neurons and in vivo on the effects of L-dopa in the 6OHDA (6-hydroxydopamine) contralateral turning model. These PAMs lacked activity at a wide variety of receptors, lacked PAM activity at related Gi-coupled G protein-coupled receptors, and lacked activity at D1 receptors. However, the PAMs did potentiate [³H]-dopamine binding at both D2 and D3 receptors. Together, these studies show that we have identified PAMs of the D2 and D3 receptors both in vitro and in vivo. Such compounds may have utility in the treatment of hypodopaminergic function.

Introduction

There has been much interest in the identification of allosteric modulators of G protein-coupled receptors (GPCRs), both as tools to understand receptor mechanisms and as potential therapeutic agents (see Keov et al., 2011; Conn et al., 2012). GPCRs represent the largest family of cell-surface receptors, and a large number of marketed drugs directly activate or block signaling mediated via these receptors. However, for some GPCRs (e.g., peptide receptors) it has proven difficult to develop small molecules; for others, achieving sufficient selectivity has been challenging due to the high degree of homology in the ligand-binding site between GPCR subtypes (e.g., dopamine D2 and dopamine D3). Accordingly, much drug research has shifted to the identification of small molecules that target sites distinct from the orthosteric natural agonist and that induce a conformational change in the GPCR, thereby allosterically modulating the receptor function.

Allosteric ligands have a diverse range of activities, including the ability to potentiate (positive allosteric modulator,

PAM) or attenuate (negative allosteric modulator, NAM) the effects of the endogenous ligand by affecting affinity and/or efficacy (Wootten et al., 2013). As well as subtype selectivity, allosteric modulators can present other potential advantages from a drug-discovery perspective such as lack of direct effect or intrinsic efficacy; only potentiating the effect of the native transmitter where and when it is released; and reduced propensity for inducing desensitization arising from constant exposure to an agonist.

The monoamine dopamine acts via two families of GPCRs to modulate motor function, reward mechanisms, central processing, and other physiologic functions. The two families are D1-like, comprising the dopamine D1 and D5 receptors, which couple to the Gs and Golf G-proteins and stimulate cAMP production; and D2-like, comprising the D2, D3, and D4 receptors which predominantly couple to Gi/o G-proteins and attenuate cAMP production (see Neve et al., 2004; Beaulieu and Gainetdinov, 2011). The D2 receptors exhibit a high degree of sequence homology with D3 receptors, and they share a predicted binding site for dopamine and other ligands (Shi and Javitch, 2002). Many therapeutic agents target the dopamine D2 and D3 receptors, notably agonists such as ropinirole, pramipexole, and rotigotine used in the treatment of motor disorders such as Parkinson's disease (Perez-Lloret

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ABBREVIATIONS: ANOVA, analysis of variance; CHO, Chinese hamster ovary; DMSO, dimethylsulfoxide; GPCR, G protein-coupled receptor; Gpp (NH)p, 5'-guanylylimidodiphosphate; GTP γ S, guanosine 5'-O-(3-thio)triphosphate; HTRF, homogenous time-resolved fluorescence; IBMX, 3-isobutyl-1-methylxanthine; L-741,626, 4-(4-chlorophenyl)-1-(1H-indol-3-ylmethyl)piperidin-4-ol; NMDA, N-methyl-D-aspartate; 6OHDA, 6-hydroxydopamine; PAM, positive allosteric modulator; PBS, phosphate-buffered saline; SB269652, N-[4-[2-(7-cyano-3,4-dihydro-1H-isoquinolin-2-yl)ethyl]cyclohexyl]-1H-indole-2-carboxamide.

and Rascol, 2010) and antagonists such as atypical and typical antipsychotic drugs (Seeman et al., 1976; Meltzer, 1999).

We therefore performed a high-throughput screen (HTS) on 80,000 compounds to identify novel allosteric modulators of the human dopamine D2 receptor. By determining changes in intracellular cAMP via homogenous time-resolved fluorescence (HTRF; CisBio Bioassays, Codolet, France) we identified three hits that exhibited PAM properties at the dopamine D2 receptor. However, one compound was a racemic mixture (1,3-benzothiazol-2-yl(2-methyl-2,3-dihydro-indol-1-yl)methanone; Fig. 1). On synthesis and chiral resolution of the two stereoisomers, one (the R stereoisomer) was shown to act as a PAM whereas the other displayed negative allosteric modulator properties. We report here the properties and characterization of these compounds in recombinant systems on both function and radioligand binding, in native tissue and in vivo in hemiparkinsonian rats.

Materials and Methods

Materials. [³⁵S]-guanosine 5'-O-(3-thio)triphosphate (GTP γ S) and [³H]-dopamine were obtained from PerkinElmer (Zaventem, Belgium). Complete protease inhibitor was obtained from Roche Diagnostics (Vilvoorde, Belgium); DNase, 3-isobutyl-1-methylxanthine (IBMX), forskolin, and apomorphine from Sigma Aldrich (Diegem, Belgium); and the cAMP dynamic range kit from Cisbio Bioassay (Codolet, France). Quinpirole and L-741,626 [4-(4-chlorophenyl)-1-(1*H*-indol-3-ylmethyl)piperidin-4-ol] were obtained from Tocris (Abingdon, United Kingdom).

Compounds were synthesized and prepared in house, dissolved in dimethylsulfoxide (DMSO) and diluted in assay buffer such that the final concentration of DMSO in the assay was 1%.

Animals and Ethics Statement. All animal experiments were performed according to the Helsinki declaration and were conducted in accordance with the guidelines of the European Community Council directive 86/609/EEC and approved by the ethics committee from UCB Biopharma SPRL (LA1220040 and LA2220363). Male Sprague-Dawley rats (Janvier, France) were housed in cages (four rats per cage) for 1 week before the experiments. They were kept on a 12-hour light/dark cycle with lights on at 06:00 and at a temperature maintained at 20–21°C and at humidity of approximately 40%. All animals had free access to standard pellet food and water before assignment to experimental groups. The animals weighed 250–275 g at the time of surgery and 400–450 g at the time of drug testing. Additional enrichment and welfare were provided (Enviro-Dri; PharmaServ, Framingham, MA) before and after the surgery. Animal health was monitored daily by the animal care staff. Surgeries were performed under ketamine and xylazine or under isoflurane anesthesia, and all efforts were made to minimize suffering. The animals were killed with CO₂ or when necessary by exsanguination.

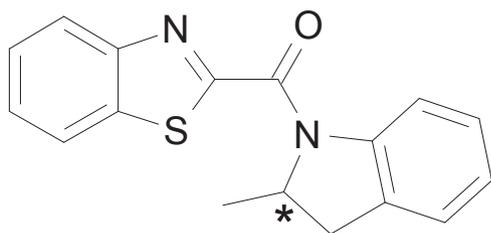


Fig. 1. Chemical structure of the dopamine D2 receptor allosteric modulator showing the racemic center.

Cell Culture and Membrane Preparation. Chinese hamster ovary cells (CHO-K1) stably expressing the human dopamine D2 long receptor or the human D3 receptor were generated in house. Human embryonic kidney cells (HEK293) transiently expressing the rat dopamine D2 long receptor were also generated in house using 293fectin (Life Technologies, Carlsbad, CA). CHO cells expressing human α_2C adrenergic receptors were obtained in collaboration with Dr. N. Moguilevsky, Free University of Brussels, Department of Applied Genetics (Brussels, Belgium). CHO cells expressing the human histamine H3 receptor were purchased from Euroscreen S.A. (Gosselies, Belgium). The cells were cultured at 37°C in a humidified atmosphere of 5% CO₂. The CHO cells were grown in Dulbecco's modified Eagle's medium/Ham's-F12 + GlutaMAX-I medium (GIBCO/Invitrogen, Gent, Belgium) containing 10% fetal bovine serum (BioWhittaker/Lonza, Vervier, Belgium), 400 μ g/ml geneticin (GIBCO/Invitrogen), and 100 IU/ml penicillin and 100 IU/ml streptomycin (Pen-Strep solution; BioWhittaker/Lonza).

Membranes were prepared from confluent cells grown in 500 cm² culture dishes. The cells were rinsed with 30 ml of phosphate-buffered saline (PBS, pH 7.4) and detached by 5 to 10 minutes of incubation in 30 ml of 1 mM EDTA in PBS at 37°C and washed with 20 ml of PBS at 4°C. The cell suspension was centrifuged at 1500g for 10 minutes at 4°C. The pellet was homogenized in 50 mM Tris-HCl (pH 7.4), 1 mM EGTA, 0.3 mM EDTA, and 2 mM MgCl₂ buffer with complete protease inhibitor, using a glass/Teflon homogenizer (10 strokes on ice at 1100 rpm). The cell pellet was then subjected to two freeze/thaw cycles, and the membranes were incubated for 10 minutes at 25°C with DNase (1 μ l/ml). The membranes were then centrifuged at 40,000g for 25 minutes at 4°C, and the final pellet was suspended in a 20 mM Tris-HCl (pH 7.4) and 250 mM sucrose buffer and stored in liquid nitrogen at a protein concentration of 2–8 mg/ml.

Radioligand Binding Assays. The CHO D2 membranes (50–100 μ g Pr per well) were incubated with [³H]-dopamine (0.05 nM to 100 nM in saturation studies; 0.6 nM in compound concentration-effect studies) for 120 minutes in 50 mM Tris buffer (pH 7.4 at 25°C, final volume 1 ml) containing (final) MgCl₂ (1 mM), pargyline (10 μ M), ascorbic acid (1 mM), and test compound or DMSO (1% to define total binding) or apomorphine (10 μ M to define nonspecific binding; Burt et al., 1975). The reaction was terminated by rapid filtration (glass fiber filters GF/B), the filters were washed 4 times with ice-cold 50 mM Tris buffer, and the retained radioactivity was determined by liquid scintillation spectroscopy. All incubations were performed in duplicate or triplicate.

The same method was used for CHO D3 membranes except that the protein concentration was 3 μ g Pr per well due to higher receptor expression. [³H]-raclopride binding to CHO D2 membranes was performed according to Wood et al. (2015).

Dopamine D2-Stimulated [³⁵S]-GTP γ S Binding. Membranes (5 μ g Pr) expressing human D2 dopamine receptors were incubated for 15 minutes at 25°C in buffer (50 mM Tris-HCl [pH 7.4] containing 3 mM MgCl₂, 50 mM NaCl, 1 μ M GDP, 10 μ g/ml saponin) that contained the test compound in a final volume of 0.2 ml. [³⁵S]-GTP γ S (0.15–0.2 nM) was then added, and the reaction continued for 60 minutes. Membrane-bound radioligand was separated by rapid filtration through glass fiber filters (GFB) and washed 4 times with filtration buffer (ice-cold 50 mM Tris-HCl [pH 7.4]), and the retained radioactivity was counted by liquid scintillation spectroscopy. All incubations were performed in duplicate or triplicate.

cAMP Cellular Assays. The effect of compounds on the levels of cAMP in the D2 CHO cells was assessed using cAMP dynamic kit 2 from CisBio Bioassays following the manufacturer's instructions. Using HTRF technology, the assay is based on competition between native cAMP produced by cells and cAMP labeled with the dye d2. The tracer binding is visualized by an anti-cAMP antibody labeled with cryptate. The assays were performed in 384 wells with 5000 cells per well in a final volume of 80 μ l. The cells were incubated with IBMX (500 μ M final), compound (varying concentrations), and forskolin (10 μ M final) in the presence and absence of varying concentrations of

dopamine with DMSO (1% final) for 60 minutes at ambient temperature. The reaction was terminated and the cells lysed, cAMP-d2 reagent and anti-cAMP antibody was added, and then it was incubated for 60 minutes at ambient temperature. The level of cAMP was then determined by measuring the fluorescence ratio (665 nm/620 nm). All incubations were performed in duplicate.

The same procedure was used for Lmtk-1 cells expressing the human dopamine D1 receptor except that forskolin was not included; the incubation was for 1 hour at room temperature with 20,000 cells per well.

Selectivity. To investigate the selectivity of any allosteric effect, compounds were evaluated at the Gi-coupled α_{2C} -adrenergic receptor and histamine H3 receptors and the related D1, D3, and D4 receptors.

Histamine-stimulated [35 S]-GTP γ S binding was performed as described in Célanire et al. (2009). Membranes (5–10 μ g proteins) expressing human H₃ receptor were incubated for 15 minutes at 25°C in 0.2 ml of a 50 mM Tris-HCl buffer (pH 7.4) containing 3 mM MgCl₂, 50 mM NaCl, 1 μ M GDP, 2 μ g saponin, 1% DMSO, and increasing concentrations of compounds for agonism/inverse agonism determination. Histamine and test compound were coincubated for 60 minutes, 0.2 nM of [35 S]-GTP γ S was added to the samples, and the incubation was continued for another 30 minutes. Assays were terminated by the addition of ice-cold 50 mM Tris-HCl buffer (pH 7.4) followed by rapid filtration and radioactivity determined as described earlier.

Binding of [3 H]-dopamine to the D3 receptor was performed as for the D2 receptor except that 3–5 μ g protein was used per well and the concentration of [3 H]-dopamine was 2–3 nM.

Dopamine-stimulated [35 S]-GTP γ S binding at the D4 receptor was performed as for the D2 receptor except that membranes were prepared from CHO cells expressing the human dopamine D4.4 receptor and that 10 μ g of protein of the membranes was used per well (Wood et al., 2015).

Dopamine-stimulated increases in cAMP at the dopamine D1 receptor in Lmtk-1 cells (Wood et al., 2015) was performed as for the dopamine D2 receptor except that 20,000 cells per well were used and that the assay did not include forskolin.

Noradrenaline-stimulated [35 S]-GTP γ S binding was performed as described earlier using 10 μ g of protein from CHO cells expressing the human α_{2C} -adrenergic receptor in Tris buffer (pH 7.4) containing 1 mM MgCl₂, 2 μ g saponin, and 1 μ M GDP.

Acutely Dissociated Striatal Neurons. Detailed procedures have been published (Cepeda et al., 1998; Flores-Hernandez et al., 2002; Cepeda et al., 2008). Adult rats were anesthetized with a mix of isoflurane and oxygen and sacrificed. Their brains were dissected and placed in ice-cold artificial cerebrospinal fluid containing (in mM) NaCl, 130; NaH₂PO₄, 1.25; NaHCO₃, 26; MgCl₂, 5; CaCl₂, 1; and glucose, 10. Coronal slices (350 μ m) were transferred to a chamber containing artificial cerebrospinal fluid (with 2 mM CaCl₂ and 2 mM MgCl₂) oxygenated with 95% O₂–5% CO₂ (pH 7.2–7.4, 290–310 mOsm, 25 \pm 2°C). The dorsal striatum was dissected and treated for 15 minutes with papain (0.5 mg/ml, Calbiochem, San Diego, CA) at 35°C in a *N*-[2-hydroxyethyl] piperazine-*N*-[2-ethanesulfonic acid] (HEPES)-buffered Hank's balanced salts solution (Sigma Aldrich) supplemented with (in mM) pyruvic acid, 1; glutathione, 0.005; NG-nitro-L-arginine, 0.1; and kynurenic acid, 1 (pH 7.4, 300–310 mOsm). The tissue was rinsed with (mM) Na isethionate, 140; KCl, 2; MgCl₂, 2; CaCl₂, 0.1; glucose, 23; and HEPES, 15. Striatal slices were mechanically dissociated and plated onto a stage of an inverted microscope.

Standard whole-cell patch clamp techniques were used to obtain voltage clamp recordings. The internal solution consisted of (in mM) *N*-methyl-D-glucamine, 175; HEPES, 40; MgCl₂, 2; EGTA, 10; phosphocreatine, 12; Na₂ ATP, 2; Na₂ GTP, 0.2; and leupeptin 0.1 (pH 7.25, 265–270 mOsm). The external solution consisted of (in mM) NaCl, 135; CsCl, 20; BaCl₂, 3; CaCl₂, 2; glucose, 10; HEPES, 10; and tetrodotoxin, 0.0003 (pH 7.4, 300–310 mOsm). Negative pressure was used to obtain tight seals (>1 G Ω), and the data were collected from neurons that had access resistances below 20 M Ω .

NMDA (*N*-methyl-D-aspartate) and quinpirole were dissolved in H₂O whereas the D2 antagonist L-741,626 and the test compound were dissolved in DMSO. All experiments contained the same final DMSO concentration (1%). Drugs were applied through a pressure-driven fast perfusion system (Warner Instruments, Hamden, CT) using an array of application capillaries synchronized by pClamp. NMDA currents (3-second duration every 15 seconds) were evoked while holding the cell at –70 mV. The D2 agonist and antagonist were both preapplied and coapplied. In separate cells, one concentration of the D2 agonist was tested before and after application of the tested compound or vehicle. The baseline for NMDA currents (100 μ M) was established, and the modulation of NMDA currents by the D2 agonist quinpirole was tested in neurons at one concentration. Quinpirole was then washed out, the test compound (10 μ M) was applied for 2 minutes before establishing a new baseline for NMDA currents, and retesting was performed with quinpirole.

6-Hydroxydopamine Lesion. To protect norepinephrinergic neurons, animals were administered imipramine HCl (Sigma Aldrich) 15 minutes before surgery. They were subsequently anesthetized with ketamine (Ceva, 75 mg/kg) and xylazine (10 mg/kg; Bayer, Diegem, Belgium) and placed in stereotaxic frame (David Kopf Instrument, Tujunga, CA). 6-Hydroxydopamine (6OHDA) was injected into the right ascending medial forebrain bundle at the following coordinates (in mm) relative to bregma and surface of the dura: anteroposterior –3.5, mediolateral –1.5, and dorsoventral –8.7. Each rat received one injection of 6OHDA HBr (4 μ g/ μ l) over a period of 5 minutes (0.5 μ l/min) for a total of 10 μ g per rat. Animals were monitored for 3 weeks to ensure full recovery and habituation to the environment and experimenters.

On day 21 after surgery, all rats were challenged with a small dose of subcutaneously administered apomorphine (0.05 mg/kg; Sigma Aldrich). Rats showing more than 90 contralateral rotations (360°) over a 45-minute recording period were included in the study.

L-Dopa methyl ester (Sigma Aldrich) was dissolved in physiologic saline solution at a volume of 5 ml/kg. UCB compound (30 mg/kg; UCB) was intraperitoneally administered as a suspension at a dose volume of 5 ml/kg in vehicle [0.1% (w/v) Polysorbate 80 (Merck, Kenilworth, NJ); 0.1% (w/v) 1510 silicone antifoam (VWR, Lutterworth, United Kingdom) in 1.0% (w/v) methylcellulose (Sigma-Aldrich, UK)]. The compound was prepared extemporaneously and homogenized by using a ultrasonic homogenizer (Covaris, Woburn, MA) and magnetic stirring.

Behavioral Recording. Vehicle or UCB compound were administered as “add-on treatment” to a subthreshold dose of L-dopa (15 mg/kg) without any dopa decarboxylase inhibitor to avoid additional potential pharmacokinetic interaction. UCB compound or vehicle were administered intraperitoneally (i.p.) 15 minutes before the L-dopa dose (15 mg/kg, i.p.).

Rotational behavior was recorded using a computerized system. Rats were fixed in a harness and linked to mechanical sensors connected directly to a computer. Each 360° clockwise or counterclockwise turn was automatically recorded for up to 120 minutes at the maximum. Throughout the experiments, rats were allocated to individual test cages.

Data Analysis. In all studies values are presented as mean \pm S.E.M. from *n* separate experiments. In the electrophysiologic studies, the data analyses were performed with Clampfit 10.3 (Molecular Devices, Wokingham, United Kingdom). The group means for all measures were compared using Student's *t* tests (for two-group comparisons) and analysis of variance (ANOVA) followed by Bonferroni *t* tests (multiple-group comparisons) using SigmaStat software (SPSS, Chicago, IL). *P* < 0.05 was considered statistically significant.

In all other in vitro assays, data analysis was performed in Prism (GraphPad Software, La Jolla, CA) using the sigmoidal dose-response equation for pharmacologic studies and one-site specific equation for saturation studies.

The efficacy of the compound to modify the level of L-dopa-induced contralateral rotations was assessed with two-way mixed ANOVA,

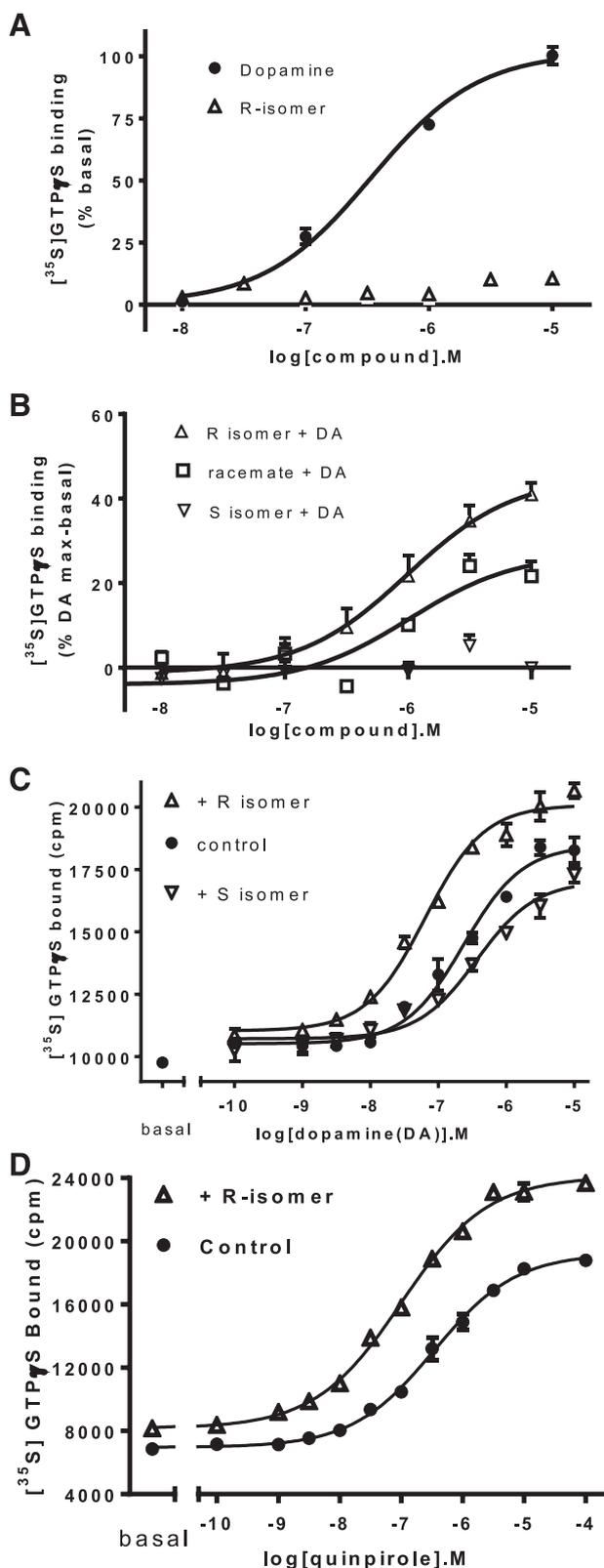


Fig. 2. Effect of compounds on D2-mediated [^{35}S]-GTP γ S binding. The results are mean and S.E.M. from triplicate determinations in a single representative experiment that was repeated twice. (A) The R isomer lacks direct effects. The response was basal in the absence of dopamine (DA). (B) The R isomer caused a greater potentiation of D2 receptor signaling in the presence of a low concentration of dopamine than the racemate in a concentration-related manner. The S isomer lacked effects. Results are normalized to fitted max DA response and minimum (basal) response in presence of EC_{20} [DA]. (C) In the presence of $10\ \mu\text{M}$ of the R isomer, the

combining the drug effect (two levels) as a between-group factor with the time (12 levels) as a within-subjects factor. Statistical analyses were performed using the Statistica software (StatSoft, Tulsa, OK). For each test, statistical significance was assumed if $P < 0.05$, and data were log-transformed before analysis.

Results

Effects on [^{35}S]-GTP γ S Binding. In membranes from CHO cells expressing the human dopamine D2 receptor, dopamine stimulated [^{35}S]-GTP γ S binding with a pEC_{50} of 6.46 ± 0.05 (mean \pm S.E.M., $n = 8$; $350\ \text{nM}$) and a stimulation of $91\% \pm 8\%$ over basal. The initial compound identified at concentrations up to $10\ \mu\text{M}$ lacked any significant effect on [^{35}S]-GTP γ S binding in the same cells on its own (6% stimulation over basal ± 3 at $10\ \mu\text{M}$, $n = 6$) (Fig. 2A). However, in the presence of a low concentration of dopamine ($30\ \text{nM}$) close to the EC_{20} , the compound now stimulated [^{35}S]-GTP γ S binding by $27\% \pm 6\%$ (fitted E_{max}) over basal (basal defined in the presence of dopamine) with a pEC_{50} of 5.99 ± 0.32 ($n = 3$; $1000\ \text{nM}$) (Fig. 2B). Upon synthesis of the stereoisomers, the R isomer stimulated [^{35}S]-GTP γ S binding in the presence of a low concentration of dopamine by $45\% \pm 3\%$ ($n = 3$) with the same pEC_{50} of 6.00 ± 0.13 whereas the S isomer lacked significant effects (Fig. 2B).

The effects of the stereoisomers on the concentration–response curve to dopamine were then investigated (Fig. 2C). At $10\ \mu\text{M}$, the R isomer significantly increased the potency (pEC_{50}) of dopamine from 6.60 ± 0.1 to 7.19 ± 0.1 ($250\ \text{nM}$ to $65\ \text{nM}$; $n = 3$, $P < 0.001$, F test; GraphPad Prism) and increased the E_{max} by 14% ($P < 0.01$, F test). In contrast, the S isomer had no effect on the potency of dopamine to 6.44 ($370\ \text{nM}$, not statistically significant) but reduced the E_{max} of dopamine by 20% ($P < 0.01$, F test).

We then examined whether the R isomer would potentiate the effects of other dopamine D2 receptor agonists. At $10\ \mu\text{M}$, the R isomer increased the potency of quinpirole (pEC_{50}) from 6.21 ± 0.08 to 6.69 ± 0.11 ($620\ \text{nM}$; mean \pm S.E.M., $n = 4$, $P < 0.01$ Student's paired t test) (Fig. 2D). Quinpirole was a full agonist compared with dopamine ($E_{\text{max}}\ 99\% \pm 2\%$ compared with dopamine fitted $E_{\text{max}} = 100\%$, mean \pm S.E.M., $n = 4$), and the presence of the R isomer at $10\ \mu\text{M}$ increased this E_{max} by $35\% \pm 2\%$ ($P < 0.01$, F test; GraphPad Prism).

We next investigated the effects of the compounds in membranes expressing the rat dopamine D2 receptor. At the rat D2 receptor, dopamine stimulated [^{35}S]-GTP γ S binding with a pEC_{50} of 6.83 ± 0.16 ($n = 5$; $150\ \text{nM}$). At the rat D2 receptor, the R isomer potentiated [^{35}S]-GTP γ S binding in the presence of a low concentration of dopamine ($10\ \text{nM}$) by $57\% \pm 6\%$ over basal (fitted E_{max} and basal with basal defined as binding in the absence of compound but presence of $10\ \text{nM}$ dopamine) with a pEC_{50} of 5.84 ± 0.06 ($n = 5$; $140\ \text{nM}$). In the absence of dopamine, there was no statistically significant effect.

Effects on cAMP at the hD2L Receptor. In the presence of forskolin, dopamine produced a concentration-dependent decrease in cAMP levels with a pEC_{50} of 9.0 ± 0.1

potency and efficacy of dopamine was increased compared with dopamine alone. The presence of the S isomer ($10\ \mu\text{M}$) had no effect. (D) In the presence of $10\ \mu\text{M}$ of the R isomer, the potency and efficacy of quinpirole was increased compared with quinpirole alone.

($n = 4$; 1 nM). In the presence of 10 μM of the racemic initial compound, the potency (pEC_{50}) of dopamine was increased to 9.6 ± 0.1 (0.25 nM) (Fig. 3A). There was no effect of the

compound on basal levels of cAMP, and there was no change in the maximum response to dopamine. We then tested the effects of the stereoisomers on the dopamine concentration–response curve (changes in cAMP assessed using the HTRF ratio; Fig. 3B). The R isomer (10 μM) produced a significant shift in the potency of dopamine pEC_{50} (EC_{50}) from 8.3 (4.6 nM) to 9.03 (0.9 nM), and the S isomer had a small but nonsignificant reduction in dopamine potency to 8.1 (8 nM). This change in apparent dopamine potency was due to the correction of raw counts to nM cAMP using the standard curve.

Effects on D2 Receptor Binding. At concentrations up to 10 μM both the R and S isomers had no statistically significant effect (<10%) on the binding of [^3H]-raclopride to the human D2 receptor (data not shown). The effects of the potential allosteric modulators on the binding of the endogenous agonist, [^3H]-dopamine, to the hD2 receptor in CHO cell membranes was then investigated. Previous literature has shown the importance of including Mg^{++} ions both in the membrane preparation and in the assay buffer (Hamblin and Creese, 1982), and in the present studies the best window was obtained using 1 mM MgCl_2 . The concentration of [^3H]-dopamine used in competition studies was selected based on signal size and also on window size (specific: nonspecific).

The specific binding window was increased in the presence of pargyline and ascorbic acid compared with that in the absence and no specific filter binding was seen. No specific binding was seen in membranes prepared from cells lacking the D2 receptor. Control compounds such as apomorphine, chlorpromazine, and (+)butaclamol produced a concentration-dependent full inhibition of [^3H]-dopamine binding to the D2 receptor with a pEC_{50} of 9.16 ± 0.04 ($n = 3$; 0.25 nM), 9.00 ± 0.50 ($n = 4$, 1 nM) and 8.58 ± 0.10 ($n = 3$; 2.6 nM), respectively. In contrast, the R isomer produced a concentration-dependent increase in [^3H]-dopamine binding (Fig. 4A). This potentiation was difficult to quantify as the response did not saturate at the concentrations tested (we could not go to higher concentrations of the compound due to solubility), but a marked 100% increase in radioligand binding was seen at 10 μM of the compound and a statistically significant effect was seen at 1 μM . The S isomer produced an inhibition of radioligand binding. Again the effect was difficult to quantify, but a statistically significant inhibition of $16\% \pm 3\%$ ($n = 3$) was seen at 3 μM compound.

The effect of the isomers on the saturation binding of [^3H]-dopamine to the dopamine D2 receptor was then studied. Analysis of the saturation binding revealed the presence of one binding site (F-test, GraphPad Prism). Saturation studies were performed in the presence and absence of 10 μM of the R and S isomers (Table 1). There was no statistically significant effect on the apparent affinity of dopamine (K_d 15 nM), but the maximum number of binding sites (B_{max} ; 3.2 ± 0.2 pmol/mg Pr) was statistically significantly increased in the presence of the R isomer ($P < 0.5$, Student's paired t test) whereas there was a small but nonsignificant reduction in the B_{max} with the S isomer (Supplemental Data). The B_{max} for the antagonist radioligand [^3H]-raclopride in the same membranes was 17 pmol/mg Pr (data not shown).

To further explore the activities of these PAM effects at the dopamine receptors, we went on to identify [5-fluoro-4-(hydroxymethyl)-2-methoxyphenyl]-(4-fluoro-1H-indol-1-yl) methanone (see the Supplemental Data for synthesis and

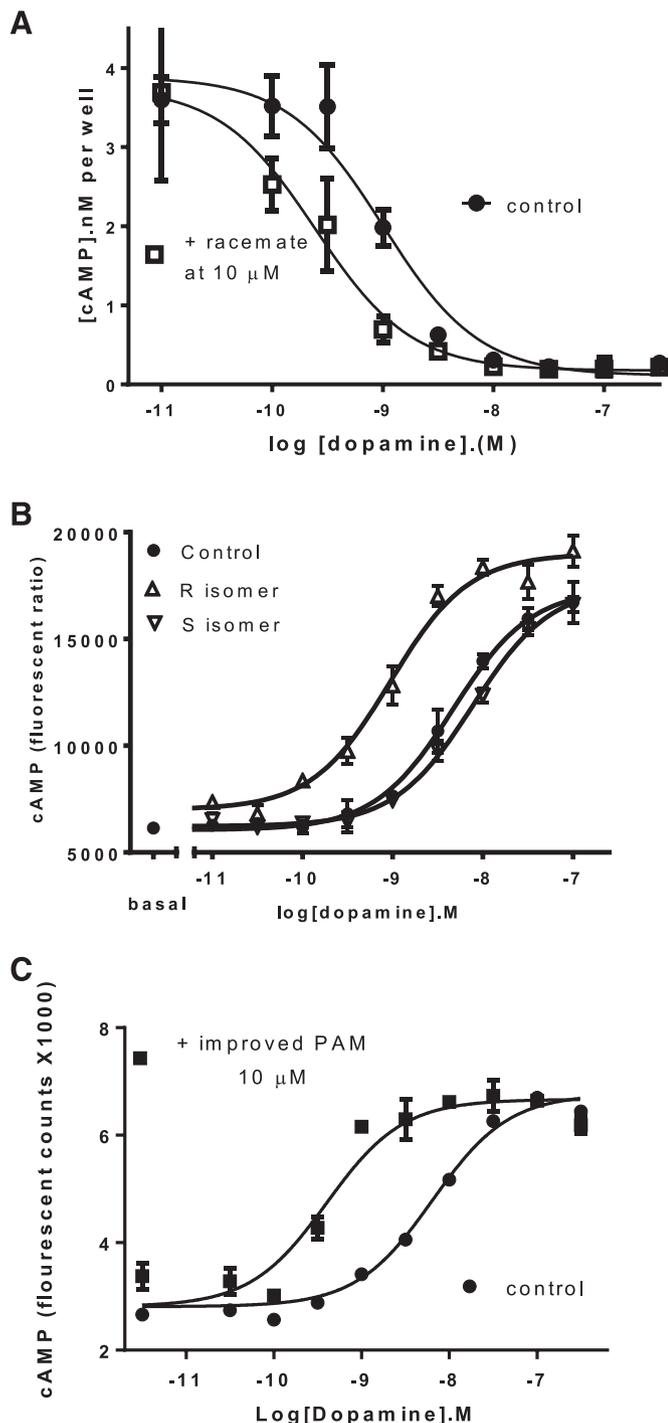


Fig. 3. Effect of compounds on D2-mediated cAMP levels. The results are mean and S.E.M. from triplicate determinations in a single representative experiment that was repeated at least twice. (A) The presence of 10 μM of the racemate increases the potency of dopamine 4-fold to inhibit cAMP production at the human dopamine D2 receptor compared with dopamine alone. Data are shown for after conversion to [cAMP]. (B) In the presence of 10 μM of the R isomer, the potency (5-fold) and efficacy of dopamine was increased compared with dopamine alone. There was a small, nonsignificant reduction in the potency of dopamine in the presence of the S isomer (10 μM). The raw data are shown in HTRF fluorescent ratio. (C) The improved compound at 10 μM increased the potency of dopamine by 15-fold.

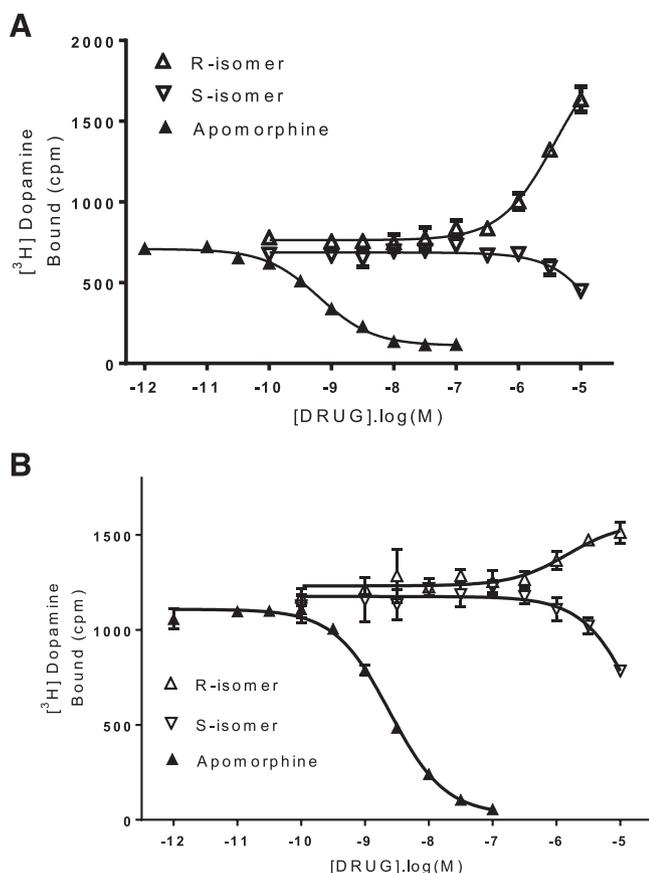


Fig. 4. Effect of compounds on [³H]-dopamine binding to the human dopamine receptors. The results are mean and S.E.M. from triplicate determinations in a single representative experiment that was repeated at least twice. (A) At the human D2 receptor, the R isomer caused a concentration-related increase in [³H]-dopamine binding whereas the S isomer decreased binding. (B) At the human D3 receptor, the R isomer caused a concentration-related increase in [³H]-dopamine binding whereas the S isomer decreased binding.

structure) as a more potent and efficacious PAM suitable for in vivo experimentation. On human D2 GTP[³⁵S]-GTPγS binding, this improved PAM lacked statistically significant effects on its own (<10% activation at 10 μM compared with maximal effect of dopamine) but potentiated the effects of a low concentration of dopamine with a pEC₅₀ of 6.2 ± 0.24 (*n* = 5; 630 nM) and by an E_{max} of 89% over basal ± 12 (compared with 57% for the R isomer and 100% for dopamine). This higher level of potentiation was evidenced by a greater shift or increase in the potency of dopamine of 15-fold (from 6.4 nM to 0.4 nM) (Fig. 4C) compared with 4-fold for the R isomer. We therefore studied the effect of the improved PAM on the saturation binding of [³H]-dopamine as earlier. This PAM (10 μM) markedly increased the number of high-affinity binding sites, giving a 3-fold increase which was markedly greater than that seen with the R isomer and consistent with its higher efficacy (Fig. 5; Table 2). The nonhydrolysable GTP analog 5'-guanylimidodiphosphate [Gpp(NH)p] (32 μM) reduced the affinity of [³H]-dopamine binding and increased the apparent number of sites. The effect of the PAM was not affected by the presence of Gpp(NH)p.

Selectivity Assays. To ensure that potentiation in dopamine mediated receptor signaling was not due to an effect at

TABLE 1

Effects of the stereoisomers on the saturation binding of [³H]-dopamine to the human dopamine D2 receptor

Data are mean ± standard deviation from three separate experiments.

	Control	+ R Isomer (10 μM)	+ S Isomer (10 μM)
K _d (nM)	15 ± 3	13 ± 3	17 ± 6
B _{max} (% control)	100 ± 6	137 ± 10*	82 ± 15

**P* < 0.05, Student's paired *t* test.

the G protein level, experiments were conducted using the human α_{2C}-noradrenergic receptor and the histamine H3 receptor. No statistically significant effects were seen at concentrations up to 10 μM on basal or agonist-stimulated [³⁵S]-GTPγS binding at either receptor for any of the compounds (data not shown, but examples shown in the Supplemental Data).

To examine selectivity further, the effects of the compounds were tested as allosteric modulators at human dopamine D1 and D3 receptors. At the D1 receptor, dopamine stimulated cAMP production with a pEC₅₀ of 8.6 ± 0.1 (*n* = 4; 2.5 nM). None of the three compounds had an effect on the D1 response (data not shown), neither on their own nor in the presence of dopamine (1.5 nM); nor did they have an effect on the dopamine concentration–response curve (Supplemental Data). In contrast, similar to their effects at the human dopamine D2 receptor, the R isomer potentiated the binding of [³H]-dopamine to the D3 receptor and the S isomer attenuated binding (Fig. 4B). The compounds also lacked activity either on their own or in the presence of dopamine at the human dopamine D4 receptor (data not shown).

Effects in Electrophysiologic Studies in Rat Striatal Neurons. At concentrations above 3 μM, quinpirole dose-dependently decreased NMDA currents (*P* < 0.0001, ANOVA, *F* 6,35 = 70.29) (Fig. 6A). The D2 antagonist L-741,626 (30 μM; Hattori et al., 2006) blocked the 10 μM quinpirole effect (not shown), demonstrating that this effect is mediated by D2 receptor activation. In the presence of the D2 antagonist, we still observed a slight decrease of NMDA currents (−13.4 ± 3.4%, *n* = 4). However, it was similar to the decrease observed with the lower concentrations of quinpirole, suggesting that the remaining decrease is induced in part by current rundown. For all further studies, the effects of compounds were therefore compared with time-matched controls.

The R isomer tested alone at 10 μM did not have a statistically significant effect on NMDA currents (−5.3 ± 1.3%, *n* = 29). In the presence of the R isomer (10 μM), the effect of quinpirole at 3, 10, 30, 100, and 300 μM (*n* = 4–11) was increased (*P* < 0.05, paired *t* test), and the potency of quinpirole pEC₅₀ was increased from 4.3 (52.5 μM) to 4.8 (15.9 μM) (Fig. 6B). As a control, incubation in 1% DMSO failed to potentiate the 3 μM quinpirole effect (*n* = 5, Fig. 6C). The PAM (R isomer) had no effect on the lowest concentration of quinpirole tested (1 μM), suggesting that any effect was not due to current rundown, which would be present at all quinpirole concentrations.

In Vivo Effects on L-Dopa Turning Behavior. The improved PAM D2 compound statistically significantly increased [*F*(1,14) = 10.45, *P* < 0.01] and prolonged [*F*(11,154) = 50.55, *P* < 0.0001] the L-dopa-induced contralateral rotations (Fig. 7). An effect of the treatment with time was also observed

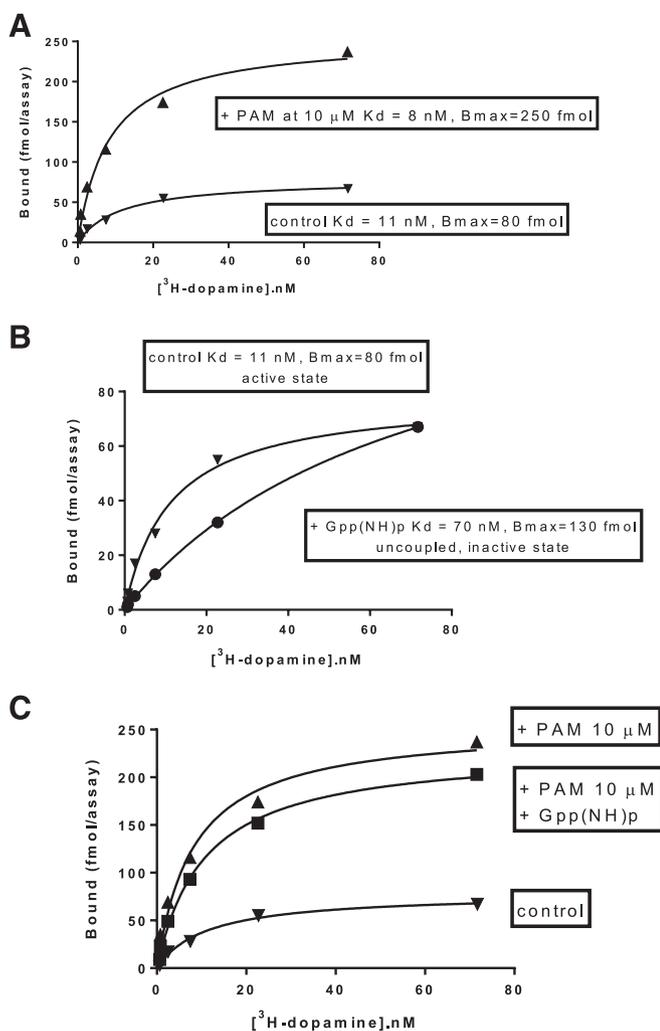


Fig. 5. Effect of [5-fluoro-4-(hydroxymethyl)-2-methoxyphenyl](4-fluoro-1*H*-indol-1-yl)methanone (10 μM) on saturation binding of [^3H]-dopamine. (A) The specific binding of [^3H]-dopamine in the presence and absence of the compound. Data from a single experiment with triplicate determination. (B) The effect of GppNHp on the saturation binding of [^3H]-dopamine. (C) The lack of effect of GppNHp on the effect of the PAM.

[$F(11,154) = 7.60$, $P < 0.0001$]. Additional statistical analysis showed that the group treated with the UCB compound had a statistically significantly higher level of contralateral rotations than the vehicle-treated group for the first 60 minutes of the test.

Discussion

We report the identification of novel allosteric modulators of the dopamine D2 receptor and D3 receptors. We identified an initial hit from an HTS that lacked agonist effects on its own (at concentrations up to 10 μM) but potentiated the effect of dopamine on the human D2 receptor in cAMP and on [^{35}S]-GTP γS binding. However, this initial hit was a mix of two isomers. We therefore separated the isomers and confirmed their conformation by X-ray—the R isomer with the methyl group up, and the S isomer with the methyl group down. Compared with the racemic mixture, the R isomer produced a greater enhancement of D2-stimulated [^{35}S]-GTP γS binding whereas the S isomer appeared inactive, suggesting that the

TABLE 2

Effect of the improved PAM on the saturation binding of [^3H]-dopamine binding to the human dopamine D2 receptor

Data are mean \pm standard deviation from three to four separate experiments.

	Control	+ PAM (10 μM)	+ Gpp(NH)p (32 μM)	+ PAM +Gpp(NH)p
K_d (nM)	18 \pm 12	11 \pm 4	87 \pm 32*	20 \pm 13
B_{max} (% control)	100 \pm 26	278 \pm 51*	200 \pm 120	289 \pm 47*

* $P < 0.05$, Student's paired t test compared with control.

presence of the S isomer in the racemate attenuated the effects of the R isomer.

We then compared the ability of the isomers to affect the concentration–response curve with the endogenous ligand dopamine. The R isomer increased both the potency and, to a lesser extent, the efficacy of dopamine, whereas the S isomer produced a small but significant reduction in the maximal response to dopamine with no effect on dopamine potency. Similar results were seen on the ability of dopamine to inhibit cAMP levels, although a reduction in maximal response to dopamine in the presence of the S isomer was not observed. This may reflect the greater receptor reserve seen in the cAMP pathway, as evidenced by the markedly higher potency of dopamine in the cAMP assay ($p\text{EC}_{50}$ 9.0) than in the [^{35}S]-GTP γS binding assay ($p\text{EC}_{50}$ 6.6).

To check that this allosteric effect was mediated at the receptor level (as opposed to an effect on the G protein), we then sought to determine whether the compounds affect other Gi-coupled receptors. Using [^{35}S]-GTP γS binding, we showed that neither compound had any significant effect as either an agonist or as a modulator at the Gi-coupled α_{2C} -adrenergic and histaminergic H3 receptors. Using the antagonist [^3H]-raclopride, we found no significant effect at concentrations up to 10 μM , indicating that the compounds did not compete at the orthosteric site. Using [^3H]-dopamine, we showed that the R isomer did not act as an orthosteric ligand to inhibit binding to the dopamine D2 receptor; rather, it increased the binding, whereas the S isomer decreased binding. The increase in [^3H]-dopamine binding was consistent with its potency on [^{35}S]-GTP γS binding, although this was difficult to quantitate because solubility of the compound precluded testing at concentrations greater than 10 μM . Similar effects on the binding of [^3H]-dopamine to the human dopamine D3 receptor were observed with both the R and S isomers.

Novel allosteric binding sites on GPCRs have been suggested to offer selectivity benefits compared with the orthosteric site, which is often highly conserved across receptor subtypes (Conn et al., 2009; Leach et al., 2010). In this study, we have shown selectivity for the D2 receptor over the D1 receptor, but not over the D3 receptor. There is a high degree of sequence similarity between the D2 and D3 receptors—over 78% within the transmembrane domains and a near identity of the residues inferred to form the dopamine-binding site. For the antagonist eticlopride, 17 of the 18 residues that form its binding site are identical across the D2 and D3 receptors (Shi and Javitch, 2002; Chien et al., 2010). Interestingly, SB269652 (*N*-[4-[2-(7-cyano-3,4-dihydro-1*H*-isoquinolin-2-yl)ethyl]cyclohexyl]-1*H*-indole-2-carboxamide) was identified as an allosteric antagonist at both the dopamine D3 and D2 receptors (Silvano et al., 2010). Recent studies have suggested that this compound acts as a bitopic ligand with

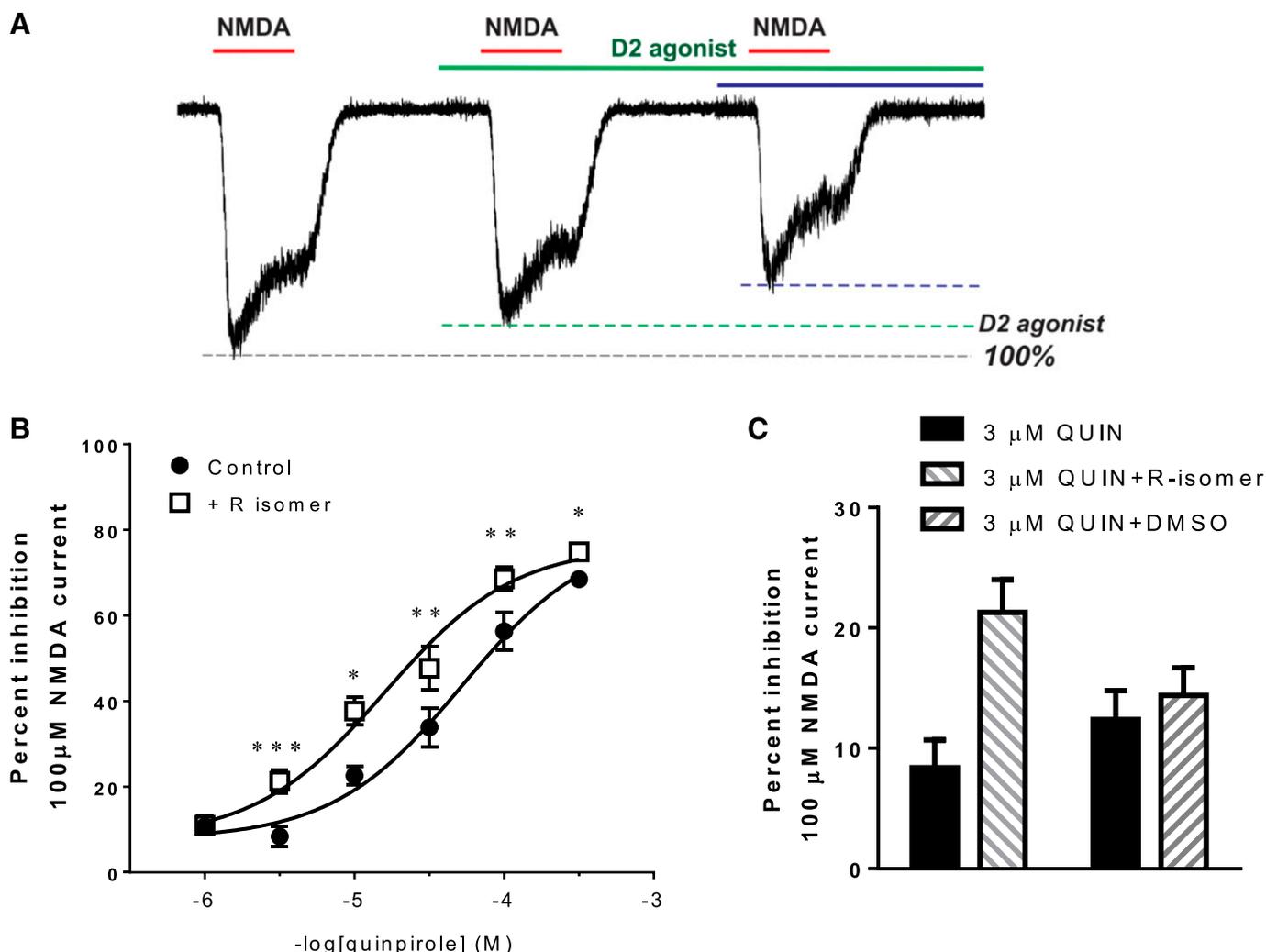


Fig. 6. (A) Traces of 100 μM NMDA-mediated calcium and barium currents before and after coapplication of 10 μM quinpirole, and before and after incubation with the R isomer. (B) Graph shows the dose–response effect of quinpirole or quinpirole + R isomer on NMDA currents. The dose–response curve was shifted to the left in the presence of PAM D2. (C) While incubation with improved PAM D2 compound potentiated the effect of 3 μM quinpirole compared with vehicle control. Paired *t* test **P* < 0.05; ***P* < 0.01; ****P* < 0.001. The blue line indicates D2 agonist + R isomer. The green line indicates D2 agonist alone.

the indole-2-carboxamide moiety interacting with an allosteric pocket (Lane et al., 2014) and has a close structural similarity to the compounds identified here. This raises the

possibility that the allosteric pocket is conserved between dopamine D2 and D3 receptors, but not across dopamine D4 or D1 receptors.

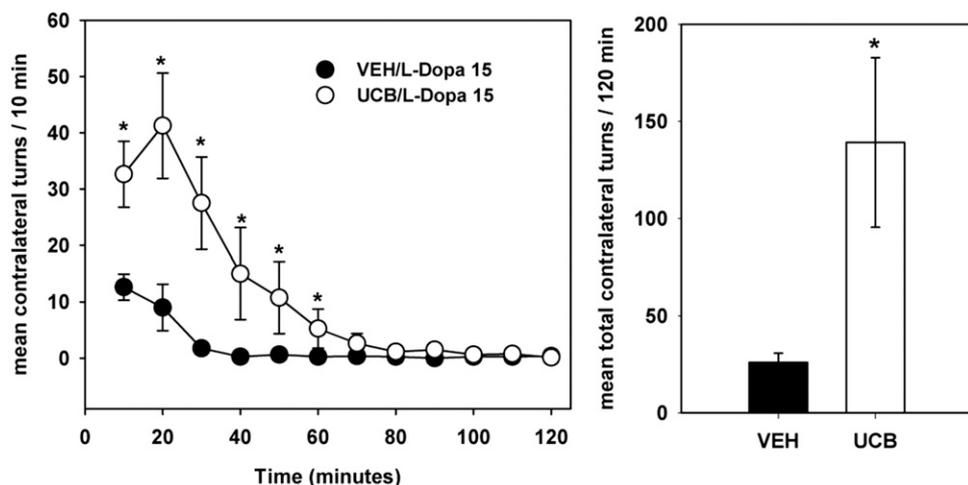


Fig. 7. Effect of improved PAM compound (30 mg/kg, i.p.) on the level of L-dopa-induced contralateral rotations in unilateral 6OHDA-lesioned rats (15 mg/kg, i.p.). **P* < 0.05: mean is significantly different from that of the vehicle-treated group (planned contrasts for every 10-minute time interval).

We had good evidence that the compounds acted as allosteric modulators at the dopamine D2 and D3 receptor, so we investigated the mechanism of this interaction by investigating their effects on the saturation binding of the orthosteric agonist [³H]-dopamine. Since the description of this assay, there have been few reports on its application, probably due to the low level of specific binding (Burt et al., 1975) compared with newer antagonist radioligands and the observation that the inclusion of Mg⁺⁺ in the membrane preparation is important (Hamblin and Creese, 1982). The affinity of [³H]-dopamine determined in the present study is in good agreement with that obtained in rat tissue (5–10 nM, Burt et al., 1975) and in CHO cells (Torvinen et al., 2004). Our data showed that the PAM did not affect the affinity of [³H]-dopamine for the D2 receptor, but instead increased the proportion of receptors in the high-affinity agonist state. This suggests that the PAM acts to enhance signaling or efficacy at the receptor. The change in potency of dopamine observed in the functional assays is probably due to a high receptor reserve leading to a ceiling in efficacy (see Kenakin, 2013). In support of this, (–)-[3H]-(3-hydroxyphenyl)-N-(1-propyl)-piperidine [(–)-3-PPP] has been reported as a partial agonist at the dopamine D2 receptor (Burriss et al., 2002) but appeared as a full agonist in the present study (data not shown). GppNHp reduced the apparent affinity for [³H]-dopamine and increased the apparent number of sites, suggesting a conversion to a low-affinity inactive state. In support of this, the number of high-affinity sites labeled by [³H]-dopamine was lower than that seen with [³H]-raclopride. The lack of effect of Gpp(NH)p on [³H]-dopamine binding in the presence of the PAM suggests that this conformational change is independent of the G protein.

As we have demonstrated robust allosteric effects at the dopamine D2 receptor, it is important to show that these effects are maintained under physiologic expression and signal transduction conditions. We show that the R isomer potentiates the effect of a dopamine D2/D3 agonist on a D2/D3 receptor-mediated response in a native tissue system, rat striatal neurons. Activation of the D2/D3 receptor induced decreases of NMDA currents in adult rat striatal neurons, and this was blocked by a dopamine D2/D3 receptor antagonist. In this assay, there was no effect of the compound alone, demonstrating a lack of agonist-like properties under physiologic expression, but it increased the potency (EC₅₀) of quinpirole from 52 to 16 μM. The potency of quinpirole (pEC₅₀ 4.28) was in agreement with other findings (Andre et al., 2010; Jocoy et al., 2011) and was consistent with a lower D2 receptor expression in the native tissue compared with the recombinant systems expressing rat D2 receptors where the pEC₅₀ for quinpirole was 6.2.

To test the hypothesis that a D2 PAM should potentiate the effects of released dopamine in vivo, we went on to identify [5-fluoro-4-(hydroxymethyl)-2-methoxyphenyl](4-fluoro-1H-indol-1-yl)methanone (Supplemental Data) as a more potent and efficacious PAM D2/D3 compound. This compound was slightly more potent, but was markedly more efficacious (almost 90% potentiation of GTPγS assay compared with 40% for the initial PAM and 3-fold shift in B_{max} of [³H]-dopamine binding compared with 1.3), and it had improved physicochemical properties resulting in sufficient brain exposure for in vivo testing.

We have used the unilateral 6OHDA lesion model in rats (Schwartz and Huston, 1996) to investigate the effects of the PAM using a threshold dose of L-dopa that induced a low level of contralateral turns. This model was expected to be sensitive to the effects of a PAM. Indeed, we saw that the compound, at 30 mg/kg, potentiated the effects of a threshold dose of L-dopa on contralateral turning in the 6OHDA model. We confirmed that this was not due to an effect on L-dopa metabolism as there was no change in the plasma exposure of L-dopa (R. Barnaby, personal communication). Unfortunately we did not have sufficient material to check the effect of the compound alone, but no overt effect of the compound was seen in the pharmacokinetic study, supporting the hypothesis that the potentiation was due to an allosteric mechanism. These data indicate that a positive allosteric modulator of the dopamine D2/D3 receptor can potentiate dopamine effects on neuronal activity in rat brain.

In conclusion, we have shown a stereoselective interaction on the allosteric modulation of the dopamine D2 and D3 receptor. The R isomer acts as a PAM in that it lacks effects on its own, but potentiates the effects of dopamine at the D2 receptor on G protein activation and cAMP signaling, and potentiates [³H]-dopamine binding to the D2 receptor. The potentiation in E_{max} seen in [³⁵S]-GTPγS binding compared with the lack of effect on the downstream signaling cAMP together with the potentiation in B_{max} and the lack of change in affinity for [³H]-dopamine, suggest that the R isomer acts as an efficacy modulator by stabilizing the active state of the receptor. In contrast, the S isomer attenuates the effects of the R isomer and inhibits [³H]-dopamine binding.

Although the evidence for an allosteric action at the dopamine D3 receptor is not as robust as that at the dopamine D2 receptor, the potentiation of [³H]-dopamine binding in the absence of an effect on the orthosteric antagonist binding is consistent with the profile demonstrated at the D2 receptor. Unfortunately, the R isomer and S isomers both produced a nonspecific effect, which interfered with our D3 receptor label-free functional assay (Wood et al., 2015) and precluded further testing.

Importantly, we show that the PAM maintains its activity in native tissue and further that this potentiation was maintained in vivo. In both these studies, an action at both dopamine D2 and D3 receptors could be involved, and further studies with selective D3 receptor antagonists are needed. The identification of positive allosteric modulators at dopamine D2 and D3 receptors could have important applications in pathologic conditions of hypodopaminergic function, such as in Parkinson's disease and restless leg syndrome.

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Authorship Contributions

Participated in research design: Wood, Andre, Ates, Gillard, Michel, Barnaby.

Conducted experiments: Andre.

Performed data analysis: Wood, Andre, Michel, Barnaby, Ates.

Wrote or contributed to the writing of the manuscript: Wood, Ates, Andre, Michel, Gillard.

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