Published in final edited form as:
Eur Neuropsychopharmacol. 2009 February ; 19(2): 138–146. doi:10.1016/j.euroneuro.2008.10.002.

Comparison of the enantiomers of (±)-doxanthrine, a high efficacy full dopamine D_1 receptor agonist, and a reversal of enantioselectivity at D_1 versus alpha$_{2C}$ adrenergic receptors

Julie A. Przybyla[a], **Juan P. Cueva**[a], **Benjamin R. Chemel**[a], **K. Joseph Hsu**[a], **David J. Riese II**[a], **John D. McCorvy**[a], **Julia A. Chester**[b], **David E. Nichols**[a], and **Val J. Watts**[a,*]

a*Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University, West Lafayette, IN 47907*

b*Department of Psychological Sciences, Purdue University, West Lafayette, IN 47907*

Abstract

Parkinson's disease is a neurodegenerative condition involving the death of dopaminergic neurons in the substantia nigra. Dopamine D_1 receptor agonists are potential alternative treatments to current therapies that employ L-DOPA, a dopamine precursor. We evaluated the pharmacological profiles of the enantiomers of a novel dopamine D_1 receptor full agonist, doxanthrine (DOX) at D_1 and α_{2C} adrenergic receptors. (+)-DOX displayed greater potency and intrinsic activity than (-)-DOX in porcine striatal tissue and in a heterologous D_1 receptor expression system. Studies in MCF7 cells, which express an endogenous human dopamine D_1-like receptor, revealed that (-)-DOX was a weak partial agonist/antagonist that reduced the functional activity of (+)-DOX and dopamine. (-)-DOX had 10-fold greater potency than (+)-DOX at α_{2C} adrenergic receptors, with an EC50 value of 4 nM. These findings demonstrate a reversed stereoselectivity for the enantiomers of DOX at D_1 and α_{2C} receptors and have implications for the therapeutic utility of doxanthrine.

Keywords

Dopamine D_1 receptors; adrenergic receptors; Parkinson's disease; enantiomeric drugs; intrinsic activity; locomotor activity

*Corresponding author, Purdue University, Dept. of Medicinal Chemistry and Molecular Pharmacology, 575 Stadium Mall Dr., RHPH 210, West Lafayette, IN 47907-2091, Tel. 765-469-3872, Fax. (765) 494-1414, wattsv@purdue.edu.

Contributors Julie Przybyla performed the pharmacological characterization of the enantiomers of doxanthrine using the heterologous D_1 and α_{2C} expression systems and MCF7 cell line. She also wrote the first draft of the manuscript and participated in the revision process. Juan P. Cueva performed the chemical synthesis of the enantiomers and racemic mixture of doxanthrine. Benjamin R. Chemel performed the studies evaluating the DOX enantiomers using the native porcine striatal tissue and α_{2C} receptor cell system and contributed to the revision process. K. Joseph Hsu performed the initial characterization of the endogenously expressed human dopamine D_1 receptor in the MCF7 cell line with the guidance of Dr. David J. Riese II. John McCorvy performed the behavioral characterization of the enantiomers of doxanthrine with the guidance of Dr. Julia A. Chester. Dr. David E. Nichols provided the chemical expertise for the design of doxanthrine and contributed to the preparation of the manuscript. Dr. Val J. Watts, the corresponding author, designed and supervised all aspects of the experimental studies and finalized the preparation of the manuscript.

1. Introduction

Parkinson's disease (PD) is a neurodegenerative condition that involves the selective degeneration of dopaminergic neurons in the substantia nigra, resulting in a dopamine deficiency in the basal ganglia, a brain area that is essential for initiation and control of voluntary movement. The degeneration of the nigrostriatal pathway leads to the classic symptoms of PD, including tremors, rigidity, and slowness of movement (Dawson and Dawson, 2003). The most widely-used treatment for PD is levodopa (L-DOPA), a dopamine precursor that crosses the blood brain barrier and is enzymatically converted into dopamine (Hurley and Jenner, 2006). Although L-DOPA is currently the "gold standard" for PD treatment, it produces both acute and long-term adverse effects and loses its efficacy in late stage PD.

Additional PD therapies include dopamine agonists that directly stimulate dopamine receptors in the striatum. The dopamine receptors are classified into two families. The dopamine D_1 receptor family, comprised of the D_1 and D_5 receptors, stimulates adenylate cyclases through coupling with the stimulatory G proteins $G\alpha_s$ and $G\alpha_{olf}$ (Huang et al., 2001). The dopamine D_2-like receptor family (D_2, D_3, and D_4 receptors) couples with inhibitory G proteins $G\alpha_{i/o}$, leading to inhibition of adenylate cyclase or modulation of other effectors (Neve et al., 2004). Currently available direct agonists for PD target the D_2-like receptors and include bromocriptine, ropinirole, and pramipexole (Hurley and Jenner, 2006; Schapira et al., 2006). Dopamine D_2 agonists appear primarily effective in early stage PD and as adjuncts to L-DOPA. Similar to L-DOPA, D_2-like receptor agonists have been shown to produce dyskinesias (Jenner, 2003; Olanow et al., 2004).

Alternative pharmacological treatments under development for treating PD have included selective dopamine D_1 receptor agonists (for review see Zhang et al, 2008). Dihydrexidine (DHX) was the first selective, full dopamine D_1 receptor agonist, and displays moderate 10-fold selectivity for dopamine D_1 versus dopamine D_2 receptors (Brewster et al., 1990; Mottola et al., 1992). Taylor et al. (1991) first demonstrated that DHX was remarkably effective at reducing MPTP-induced Parkinson-like symptoms in monkeys. Additionally, in a small human patient trial ABT431, another selective D_1 agonist, was demonstrated to be as efficacious as L-DOPA at alleviating the symptoms of PD (Rascol et al., 2001). These studies confirmed, contrary to conventional wisdom, the importance of the dopamine D_1 receptor as a potential target for PD.

We recently described the synthesis and preliminary characterization of doxanthrine (DOX), a bioisostere of DHX that has improved selectivity for dopamine D_1 receptors (Cueva et al., 2006). Racemic DOX displayed approximately 100-fold greater selectivity for D_1 over D_2 receptors (Cueva et al., 2006). We found that (+)-DOX displayed 200-fold selectivity for D_1 over D_2, whereas (-)-DOX possessed only 20-fold selectivity for D_1 versus D_2 receptors. These studies revealed that (+)-DOX is a potent full agonist at the recombinant human D_1 dopamine receptor. Here we have extended our studies to examine the functional activity of both the (+) and (-) enantiomers of DOX using porcine D_1-like receptors as well as a novel endogenous human D_1-like dopamine receptor model. To validate our biochemical studies, we used an in vivo mouse model to investigate the biological activity of both the (+) and (-) enantiomers of DOX to produce changes in locomotor activity.

In addition, the initial study by the NIMH-sponsored Psychoactive Drug Screening Program revealed that racemic DOX possesses significant binding affinity for the α_{2C} adrenergic receptor (Cueva et al., 2006). In view of the fact that α_2 adrenergic receptors are responsible for control of blood pressure, blood flow, and neurotransmitter release (Brede et al., 2004; Docherty, 1998), it seemed likely that racemic DOX might have additional undesirable

pharmacological targets. Thus, we also examined the functional properties of (±)-, (+)-, and (-)-DOX at the α_{2C} adrenergic receptor using a recombinant heterologous system.

In this work we demonstrate that (+)-DOX is a potent dopamine D_1 receptor agonist in several systems. By contrast, (-)-DOX is a weak partial agonist at D_1 receptors, but exhibits potent *agonist* activity at α_{2C} receptors. Thus, enantioselectivity for activity at the α_{2C} receptor is reversed compared to the D_1-like (and D_2-like) receptors (Cueva et al., 2006). This finding is remarkable in view of the fact that the D_1, D_2, and α_2 receptors are all Family A G protein-coupled receptors, presumably with a common rhodopsin-like structure, and has theoretical relevance to understanding the binding motifs of agonist ligands in these receptors. Of more practical significance, however, is the demonstration that (+)-DOX has clear superiority over racemic DOX as a potential therapeutic agent, where the off-target α_{2C} adrenergic receptor activation of (-)-DOX in the racemate would produce unwanted side effects. This interesting example is illustrative of a case where the less active enantiomer in a racemate is not simply inert, or “less active,” but possesses an active pharmacology that diverges from that of its antipode.

2. Materials and Methods

2.1. Chemicals and Reagents

[^{3}H] Cyclic AMP (30 Ci/mmol) was purchased from PerkinElmer (Boston, MA, USA). Dopamine, clonidine, SCH-23390, SKF38393, and isobutylmethylxanthine were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Forskolin was purchased from Tocris Bioscience (San Diego, CA, USA). Enantiomers of DOX were synthesized as described previously (Cueva et al. 2006). pcDNA3.1/V5/his TOPO human D_1 dopamine receptor was a gift from Dr. Bryan Roth and pcDNA3.1-α_{2C} was provided by Missouri S&T cDNA Resource Center (www.cdna.org).

2.2. Production of Cell Lines

HEK-α_{2C} cells were constructed by stable transfection of HEK 293 cells with pcDNA-3.1(+)-α_{2C}. G418 resistant clones were selected and assayed for α_{2C} function by measuring clonidine-mediated inhibition of forskolin-stimulated cyclic AMP accumulation. HEK-CreLuc cells were constructed by stable transfection of HEK 293 cells with pGL3, which contains the luciferase (Luc) gene under the transcriptional control of five copies of the cyclic AMP response element (CRE). HEK-D_1 cells were constructed by stable transfection of HEK-CreLuc cells with pcDNA3 V5 HisTopo-hD_1. Clones were assayed for D_1 receptor binding using [^{3}H] SCH23390 and for function by measuring dopamine-stimulated luciferase activity.

2.3. Cell Culture

HEK-α_{2C} cells were maintained in DMEM with 5% Fetalclone I, 5% bovine calf serum, 0.05 μg/ml penicillin, 50 μg/ml streptomycin, 25 μg/ml amphotericin B, and 300 μg/ml G418. MCF7 cells were maintained in MEM with 10% Fetalclone III, 1.0 mM sodium pyruvate, 0.01 mg/ml insulin, 0.05 μg/ml penicillin, 50 μg/ml streptomycin, and 25 μg/ml amphotericin B (Pitfield et al., 2006). HEK-D_1 cells were maintained in DMEM with 5% Fetalclone I, 5% bovine calf serum, 0.05 μg/ml penicillin, 50 μg/ml streptomycin, 25 μg/ml amphotericin B, 300 μg/ml G418, and 2 μg/ml puromycin. Cells were grown at 37 °C in a humidified incubator with 5% CO_2.

2.4. Cyclic AMP accumulation assay

Assays were performed on confluent monolayers of cells in 48-well plates. All drugs were diluted in Earle’s balanced salt solution (EBSS) assay buffer (EBSS containing 2% bovine calf

serum, 0.025% ascorbic acid, and 15 mM HEPES, pH 7.4) and added on ice. Cyclic AMP accumulation assays were performed by incubating the cells with ligands for 15 minutes at 37 C. Assays were performed on HEK-α_{2C} cells in the presence 30 μM forskolin (to stimulate cyclic AMP accumulation) and 1 μM SCH23390 to preclude activation of low levels of endogenous D_1-like dopamine receptors. All assays were performed in the presence of 500 μM isobutyl-methylxanthine (IBMX) and terminated with ice-cold 3% trichloroacetic acid.

2.5. Cyclic AMP binding assay

Cylic AMP accumulation was quantified using a previously described protocol (Watts and Neve, 1996). Briefly, trichloroacetic acid extracts (10-20 μL) were added in duplicate to cyclic AMP binding buffer (100 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA) in assay tubes containing 1 nM [^{3}H] cyclic AMP (final concentration) and bovine adrenal gland cyclic AMP binding protein (100-150 μg in 500 μl binding buffer). The binding assay was incubated on ice at 4 °C for 2-4 h and terminated by harvesting with ice cold wash buffer (10 mM Tris, 0.9% NaCl) using a 96-well Packard Filtermate cell harvester and Multiscreen Harvest Plates from Millipore (Billerica, MA, USA). Packard Microscint O (40 μL) was added to each well after drying. Radioactivity was counted using a Packard Topcount scintillation counter. Standard curves ranging from 0.01 to 300 pmol of cyclic AMP were used to determine the concentration of cyclic AMP in each sample. Data analysis was performed using GraphPad Prism software. Dose response curves for cyclic AMP accumulation were analyzed using nonlinear regression and data were fit to a sigmoidal dose-response curve (slope = 1) to provide estimates for EC50 and intrinsic activity values.

2.6. Porcine Striatal Adenylate Cyclase Assay

Fresh porcine brain tissue was provided by the Purdue Butcher Block. Striatal tissue was isolated by dissection and suspended in nine volumes of homogenization buffer (20 mM Hepes, 0.32 M sucrose, pH 7.4), followed by homogenization using 10-15 strokes with a Wheaton-Teflon glass homogenizer. The resulting mixture was centrifuged at 1,000 × g for 10 min at 4 °C. The pellet was washed in 10 mL of homogenization buffer and centrifuged again at 1,000 × g for 10 min at 4 °C. The resulting supernatants were combined and centrifuged at 30,000 × g for 10 min at 4 °C. The pellet was resuspended in 20-100 mL of 50 mM Tris buffer (pH 7.4) by briefly using a Kinematica homogenizer and was centrifuged at 30,000 × g for 30 min at 4 °C. This pellet was resuspended again in 50 mM Tris buffer, dispensed into 1 mL aliquots, and centrifuged for 10 min at 13,000 × g and 4 °C. A BCA protein assay was used to determine the final protein concentration of the pellets. The supernatant was aspirated and the pellets were frozen at -80°C until use.

The striatal adenylate cyclase assay protocol was adapted from previously published methods (Chester et al., 2006). Assays were carried out in 96-well assay tubes containing (final concentration) reaction buffer (5 mM $MgCl_2$, 2 mM EDTA, 1 mM IBMX, 0.01% ascorbic acid, 10 μM pargyline, and 15 mM HEPES, pH 7.4), 20 μL reaction mix (1.25 mM adenosine 5'-triphosphate (ATP), 21.5 mM N-[imino(phosphonoamino)methyl]-N-methylglycine disodium salt (phosphocreatine), and 3 U creatine phosphokinase), 1 μM Gpp(NH)p, 30 μg striatal protein, and the indicated drugs, all in a total volume of 100 μL. Duplicate samples for each treatment were incubated in a 30 °C water bath for 15 min. Adenylate cyclase activity was terminated by the addition of 200 μL of 3% trichloroacetic acid. The reaction tubes were covered with parafilm and stored at 4°C until the concentration of cyclic AMP was quantified as described above.

2.7. Mouse Locomotor Activity

Drug naïve male Swiss-Webster mice (Harlan, Indianapolis, IN) were housed in groups of 3-5 with free access to food and water under a 12/12 h light/dark cycle (lights on at 0700). All mice

weighed between 25 and 35 grams and were approximately 8-9 weeks of age at the start of the experiment. Experimental procedures were conducted between 0800 and 1200.

Locomotor activity was monitored using an open field activity frame (SmartFrame Low Density, Lafayette Instrument Co, Lafayette, IN) that contained eight infrared photo beams along the long axis and four along the short axis of each frame [internal frame dimensions: 24.13 × 45.72 centimeters (cm)]. The frames surrounded a Plexiglas chamber that resided on top of black polyurethane enamel coated metal trays. Hamilton-Kinder MotorMonitor (Model HMM100) software was used to monitor activity.

Mice were randomly assigned to one of five treatment groups: vehicle/vehicle (n=8); vehicle/(-)-DOX (n=8); vehicle/(+)-DOX (n=8); SCH23390/(+)-DOX (n=8); SCH23390/vehicle (n=6). For activity testing, individual mice were weighed and placed in the locomotor activity chambers for 30 minutes to acclimate them to the testing environment. Mice were then removed from the chamber and injected with either 0.03 mg/kg SCH23390 or vehicle. After a 20 min pretreatment time, mice were subsequently injected with either 5.0 mg/kg (+)-DOX, 5.0 mg/kg (-)-DOX, or vehicle, and placed into the locomotor activity chambers for 60 min. All drugs were dissolved in physiological saline with 0.02% ascorbic acid to prevent drug oxidation and were injected intraperitoneally in a volume of 10 mL/kg body weight. Activity data were collected in 5-min epochs and data presented represent total distance traveled (cm) during the first 30 min of the testing session. Data were analyzed using a one-way ANOVA followed by post-hoc Student Newman-Keuls test using SPSS 15.0.1.1 and GraphPad Prism software.

3. Results

3.1. (+)-DOX is a potent agonist at the dopamine D_1 receptor

We initially compared stimulation of cyclic AMP accumulation by the enantiomers of DOX using a heterologous expression system of HEK cells that stably express the human dopamine D_1 receptor. Consistent with our previous studies (Cueva et al., 2006), both enantiomers stimulated cyclic AMP accumulation in this system. The (+) enantiomer of DOX displayed full intrinsic activity (111 ± 3 %) relative to dopamine with an EC50 of ca. 50 nM (Figure 1). The (-) enantiomer of DOX displayed markedly reduced potency with a moderate reduction in intrinsic activity when compared to either (+)-DOX or dopamine (Figure 1).

Knowledge that the functional activity of D_1 receptor agonists can be distorted in the presence of spare receptors in heterologous systems (Watts et al., 1995) prompted additional experiments in cells expressing an endogenous human D_1-like dopamine receptor. For these experiments we took advantage of cell growth studies implicating the presence of a D_1-like receptor in the MCF7 human breast cancer tumor cell line (Johnson et al., 1995). To initiate these studies, we completed a characterization of the human dopamine D_1-like receptor using the well-studied full D_1 receptor agonist DHX and the partial D_1 receptor agonist, SKF38393. These experiments revealed that dopamine, DHX, and SKF38393 stimulated cyclic AMP accumulation in a dose-dependent manner in MCF7 cells, with EC50 values of 1120 ± 100 nM, 81 ± 1 nM, and 1060 ± 290 nM, respectively (n = 3). DHX was a “full” agonist relative to dopamine, whereas the selective partial agonist, SKF38393 displayed the anticipated reduced intrinsic activity (ca. 30% relative to dopamine) (Figure 2A). The intrinsic activities of DHX and SKF38393 in MCF7 cells studies are consistent with previous studies using human striatal tissue (Gilmore et al., 1995). To characterize this cell model further, we carried out antagonist studies using the dopamine D_1 antagonist, SCH23390 (Figure 2B). Incubation with SCH23390 resulted in a complete blockade of agonist-stimulated cyclic AMP accumulation, indicating the presence of a functional human dopamine D_1-like receptor in MCF7 cells.

Having established MCF7 cells as a model for assessing D_1 agonist activity, the functional properties of the enantiomers of DOX were then evaluated. Studies with racemic (±)-DOX revealed that it was more potent than dopamine; however, the intrinsic activity appeared to be slightly reduced compared to dopamine (Figure 3A). In results that were consistent with those from the heterologous expression system, (+)-DOX displayed intrinsic activity that appeared to be greater than that of dopamine, suggesting that (+)-DOX may have greater efficacy than dopamine itself. In contrast, (-)-DOX had only 30% intrinsic activity when compared to dopamine. This finding suggests that (-)-DOX is a partial agonist and would have antagonist activity at dopamine D_1 receptors (Figure 3A). Thus, we evaluated the ability of (-)-DOX to antagonize dopamine- and (+)-DOX-stimulated cyclic AMP accumulation in MCF7 cells by measuring dose-response curves in the absence or presence of 10 μM (-)-DOX. As anticipated, the addition of 10 μM (-)-DOX reduced the intrinsic activity and potency of both dopamine and (+)-DOX (Fig. 3B,C). The (-)-DOX-induced reduction in the intrinsic activity of (+)-DOX indicates that (-)-DOX contributes significant antagonist activity within racemic DOX, consistent with the results shown in Figure 3A. Curiously, as shown in figures 3B,C, increasing concentrations of either dopamine or (+)-DOX failed to overcome the attenuated cAMP response produced by (-)-DOX. These results suggest that (-)-DOX may be acting either as a noncompetitive antagonist, or perhaps as a negative allosteric modulator of the D_1 receptor.

In the absence of human striatal tissues to study a native D_1-like dopamine receptor, (+)-DOX was evaluated and compared to dopamine and SKF383983 at D_1-like dopamine receptors in porcine striatal tissue. This series of functional studies revealed that (+)-DOX had high intrinsic activity (115 ± 15%; n = 3) and an EC50 of 68 ± 14 nM; n = 3 (Figure 4). Consistent with results obtained using the heterologous expression system and MCF7 cells, (+)-DOX also was more potent than dopamine, which had an EC50 value of 370 ± 77 nM (n = 3) in porcine striatal tissue.

3.2. (-)-DOX is a potent agonist at the α_{2C} adrenergic receptor

The NIMH-sponsored Psychoactive Drug Screening Program assessed racemic DOX and reported that it possessed significant affinity for α_2 adrenergic receptors (Cueva et al., 2006). The estimated Ki values for α_{2A}, α_{2B}, and α_{2C} receptors were 180, 10, and 2 nM, respectively. In light of these observations, we evaluated the functional activity of the enantiomers of DOX at the α_{2C} adrenergic receptor. A heterologous expression system in which HEK293 cells stably expressed the α_{2C} receptor was constructed to examine the ability of (+)- and (-)-DOX to inhibit forskolin-stimulated cyclic AMP accumulation. The prototypical α_2 agonist clonidine was used for comparison. Surprisingly, (-)-DOX had an EC50 value of 4.4 ± 2.3 nM (n = 3) in these cells, was nearly four-fold more potent than clonidine (EC50 = 17 ± 3.2 nM, n = 3), and was 30-fold more potent than (+)-DOX (EC50 = 151 ± 25 nM, n = 3) (Figure 5). Perhaps more striking is the marked intrinsic activity difference between the two enantiomers of DOX at the α_{2C} receptor. (-)-DOX exhibited intrinsic activity similar to that of clonidine, whereas (+)-DOX had very low intrinsic activity (34 ± 7% inhibition) that was only ca. 50% of that displayed by (-)-DOX. Moreover, the addition of a selective α_2 antagonist, rauwolscine (10 μM) antagonized the (-)-DOX- and clonidine-mediated inhibition of forskolin stimulation of cyclic AMP, demonstrating that this effect was specific for the activation of the α_{2C} receptor (data not shown). These results clearly show that the data from the NIMH-sponsored affinity studies of α_{2C} adrenergic receptors with racemic DOX primarily reflect the binding of (-)-DOX. Additionally, we reveal for the first time that the stereoselectivity of the enantiomers of the rigid D_1 agonist DOX is reversed between dopamine D_1 and α_{2C} adrenergic receptors.

3.3. (+)-DOX and (-)-DOX produce opposite effects on locomotor activity

When administered alone, (+) and (-)-DOX produced opposite effects on locomotor activity (Figure 6). One-way ANOVA of total distance traveled during the first 30 min revealed a

significant difference between treatment groups ($F_{4,33} = 7.0$, $p < 0.001$). Student Newman-Keuls post-hoc analysis indicated that (-)-DOX decreased distance traveled compared to the vehicle group ($p < 0.05$), whereas (+)-DOX increased distance traveled compared to the vehicle group ($p < 0.05$). Pretreatment with the D_1- selective antagonist, SCH23390 (0.03 mg/kg, i.p., 20 min pretreatment) blocked the locomotor-stimulatory effects of (+)-DOX. SCH23390 administered alone did not alter locomotor activity when compared to the vehicle group (Figure 6).

4. Discussion

DOX is a recently synthesized analogue of DHX, a full dopamine D_1 agonist with anti-parkinsonian activity in MPTP-treated non human primates (Cueva et al., 2006; Taylor et al., 1991). In the present studies, we have examined the pharmacological properties of the enantiomers of DOX at dopamine D_1 and α_{2C} adrenergic receptors. Initial experiments compared the functional activity of (+)-DOX and (-)-DOX, as well as the endogenous agonist dopamine, at the D_1 dopamine receptor using a heterologous D_1 expression system (HEK-D_1 cells), a novel cell model system that endogenously expresses a D_1-like receptor (MCF7 cells), and porcine striatal tissue. The (+) enantiomer of DOX displayed full agonist properties and was more potent than dopamine and (-)-DOX in all three systems. The ability of (+)-DOX to increase locomotor activity also is consistent with a D_1-like activation profile (Desai et al., 2005). It should be noted, however, that the effects of (+)-DOX on locomotor activity in the present study are quite modest when compared to the robust increase observed in response to amphetamine (Villarreal et al., 1973).

In contrast, functional studies using the α_{2C} adrenergic receptor revealed that (-)-DOX was more potent than (+)-DOX. Furthermore, (-)-DOX also was four-fold more potent than clonidine at activating α_{2C} adrenergic receptors. Consistent with these results, (-)-DOX produced a reliable decrease in locomotor activity in mice within the first 30 minutes of administration (Figure 6). This result is consistent with studies using other α_2 agonists such as clonidine, which produce distinct suppression of locomotor activity in rodents (Capasso and Loizzo, 2001).

This reversal of stereoselectivity has implications for the use of DOX as a potential therapeutic agent. For example, administration of (+)-DOX, as opposed to the racemic mixture, would allow for full stimulation of the dopamine D_1-like dopamine receptor in the absence of potentially significant adverse side effects associated with activation of the α_{2C} receptor. In addition to possessing disparate pharmacology, the two enantiomers may have different pharmacokinetic and pharmacodynamic properties due to stereoselective interaction with other molecular targets (Waldeck, 2003).

Fundamentally, the development of a single enantiomeric form of a drug is to minimize the xenobiotic load on the organism by reducing the levels of foreign molecules not normally present (Ariens, 1990). The development of therapeutic agents that are enantiomerically pure also will minimize the potential for off target effects by the “inactive” enantiomer. For example, one isomer of the drug may possess a desirable physiological and pharmacological response, whereas the other potentially could produce adverse effects by activation of other biological targets or enzymes. This situation is not what is typically observed but is, however, the result observed in our studies with the enantiomers of DOX.

Common examples of enantiomeric drugs include (*S*)-citalopram (Escitalopram or Cipralex™), a member of the SSRI antidepressant family (Izake, 2007). Escitalopram is a selective serotonin reuptake inhibitor in which the *S* enantiomer is 150 times more potent than the *R* enantiomer as a serotonin transporter inhibitor (Hyttel et al., 1992). Another example of

a single enantiomer drug is Lunesta™ (Eszopiclone, or (*S*)-zopiclone), a nonbenzodiazepine hypnotic agent used to treat insomnia. Although the mechanism of action of Eszopiclone is not well understood, the *S* enantiomer has higher affinity and is more active at GABA receptors (Blaschke, 1993). Thus, in a study where the behavioral effects of the enantiomers of zopiclone were evaluated in rats, both (*S*)- and racemic zopiclone reduced locomotor activity, whereas (*R*)-zopiclone did not, showing that the sedative effects are mediated solely by the *S* enantiomer (Carlson et al., 2001). In neither of these examples, however, was there any evidence that the enantiomer with lower potency for the desirable therapeutic response produced any adverse effects.

By contrast, in this study we have shown that (-)-DOX, which has lower potency and intrinsic activity as an agonist at the D_1 dopamine receptor, actually is a potent agonist with high intrinsic activity at α_{2C}-adrenergic receptors, with potential adverse actions such as sedation and effects on blood pressure resulting from α_2 receptor activation. Further, the indication that (-)-DOX may be a noncompetitive antagonist or negative allosteric modulator of the D_1 receptor suggests that the combination of enantiomers, in the form of the racemate, would be detrimental to therapeutic efficacy. In essence, one could consider the enantiomers of DOX to be completely different pharmacological entities.

PD patients experience significant relief in response to initiation of L-DOPA drug therapy. The onset of dyskinesias, however, an impairment of voluntary movement, is a side effect that commonly develops upon long-term treatment with L-DOPA. Moreover, the effectiveness of L-DOPA eventually declines as the disease progresses (Olanow et al., 2004). Clinically available alternatives to L-DOPA are based on the hypothesis that the therapeutic effects of L-DOPA are mediated through dopamine D_2-like receptors. Despite that belief, however, D_2 dopamine agonist monotherapy is not as efficacious as L-DOPA at alleviating PD-like symptoms. The lack of D_2 agonist efficacy as a monotherapy when compared with L-DOPA indirectly supports the role of D_1-like receptors in PD therapy. More direct evidence, however, is the demonstration that the selective D_1 agonists DHX, ABT 431, and CY208-243 reduced PD-like symptoms in a non-human primate model (Taylor et al., 1991; Temlett et al. 1988), as well as PD symptoms in humans (Rascol et al., 2001; Tsui et al., 1989), with efficacy comparable to L-DOPA, thereby demonstrating the importance of D_1 receptors as targets for the therapy of PD.

Dopamine agonists may have additional therapeutic roles beyond the relief of PD symptoms, including neuroprotective effects that lead to neuronal survival (Lewis et al., 2006). The partial D_1 dopamine agonist SKF38393 reduces aspects of MPTP-neurotoxicity (Muralikrishnan and Ebadi, 2001) and protects against malonate-induced lesions (Fancellu et al., 2003). Additionally, dopamine treatment resulted in a reduction of apoptosis in cells expressing both D_1 and D_2-like receptors (Colombo et al., 2003). This effect was blocked by the D_1-selective antagonist, SCH23390, but not by the D_2-selective antagonists haloperidol or domperidone, thereby suggesting that activation of D_1-like receptors is anti-apoptotic.

Dopaminergic neuronal degeneration and the subsequent dopamine deficit can result in a characteristic impairment of cognition and working memory in individuals with PD (Castner and Goldman-Rakic, 2004). Stimulation of the D_1 receptor enhances working memory in humans, dopamine-deficient monkeys, and aged monkeys (Arnsten et al., 1994; Cai and Arnsten, 1997; Castner and Goldman-Rakic, 2004; Davidson et al., 1990; Schneider et al., 1994). Monkeys exposed to low doses of MPTP, a neurotoxin that targets and results in the death of dopaminergic neurons, develop severe cognitive dysfunction. Administration of DHX resulted in a dose-dependent improvement of working memory and this effect was blocked by the D_1 receptor antagonist SCH-23390 (Schneider et al., 1994). Dopaminergic deficits also can occur during the natural aging process, resulting in impairment of short and long term

memory. Castner and Goldman-Rakic (2004) demonstrated that aged rhesus monkeys (20-30 + years of age) displayed a significant improvement in working memory task performance following administration of the D_1 receptor agonist ABT-431. In addition, this enhancement in cognitive function persisted for more than one year after ABT-431 administration, suggesting that stimulation of D_1 receptors may induce long-term alterations in the neurocircuitry of working memory. Most recently, a clinical trial of DHX in schizophrenia has shown that a single dose can significantly enhance blood flow to the prefrontal cortex, a brain area implicated in working memory (Mu et al., 2007). Although the clinical application of D_1 agonists (e.g. DHX) has been hampered by low bioavailabilty, our recent *in vivo* studies using the 6-OH lesioned rotating rat model suggest that DOX is orally bioavailable (McCorvy et al., unpublished observations).

In conclusion, the present study has demonstrated that (+)-DOX is a potent full D_1 dopamine receptor agonist that has high selectivity for D_1-like dopamine receptors. In contrast, (-)-DOX is a weak partial D_1 agonist with potential D_1 antagonist properties, also having significant affinity and potency at α_{2C} adrenergic receptors. Based on these results, we propose that the (+) enantiomer of DOX is an improved tool for the continued study of D_1 dopamine receptor function *in vivo*. Additionally, such a selective compound offers potential for examining the role of D_1-like receptors in the therapy of Parkinson's disease, neuroprotection, and cognitive impairment.

Acknowledgements

This work was supported by the Showalter Trust Fund Award to DJR and VJW, a TRASK award from Purdue University, NIMH grants MH060397 (VJW) and MH42705 (DEN), and the NIMH PDSP program.

References

Ariens EJ. Racemic therapeutics--ethical and regulatory aspects. E J Clin Pharmacol 1991;41:89–93.

Arnsten A, Cai J, Murphy B, Goldman-Rakic P. Dopamine D1 receptor mechanisms in the cognitive performance of young adult and aged monkeys. Psychopharmacol 1994;116:143–151.

Blaschke G, Georg Hempel, Müller Walter E. Preparative and analytical separation of the zopiclone enantiomers and determination of their affinity to the benzodiazepine receptor binding site. Chirality 1993;5:419–421. [PubMed: 8398600]

Brede M, Philipp M, Knaus A, Muthig V, Hein L. alpha2-adrenergic receptor subtypes - novel functions uncovered in gene-targeted mouse models. Biol Cell 2004;96:343–348. [PubMed: 15207902]

Brewster WK, Nichols DE, Riggs RM, Mottola DM, Lovenberg TW, Lewis MH, Mailman RB. trans-10,11-dihydroxy-5,6,6a,7,8,12b-hexahydrobenzo[a]phenanthridine: a highly potent selective dopamine D1 full agonist. J Med Chem 1990;33:1756–1764. [PubMed: 1971308]

Cai JX, Arnsten AF. Dose-dependent effects of the dopamine D1 receptor agonists A77636 or SKF81297 on spatial working memory in aged monkeys. J Pharmacol Exp Ther 1997;283:183–189. [PubMed: 9336323]

Capasso A, Loizzo A. Clonidine-induced antinociception and locomotor hypoactivity are reduced by dexamethasone in mice. J Pharm Pharmacol 2001;53:351–360. [PubMed: 11291750]

Carlson JN, Haskew R, Wacker J, Maisonneuve IM, Glick SD, Jerussi TP. Sedative and anxiolytic effects of zopiclone's enantiomers and metabolite. Eur J Pharmacol 2001;415:181–189. [PubMed: 11274997]

Castner SA, Goldman-Rakic PS. Enhancement of working memory in aged monkeys by a sensitizing regimen of dopamine D1 receptor stimulation. J Neurosci 2004;24:1446–1450. [PubMed: 14960617]

Chester JA, Mullins AJ, Nguyen CH, Watts VJ, Meisel RL. Repeated quinpirole treatments produce neurochemical sensitization and associated behavioral changes in female hamsters. Psychopharmacol 2006;188:53–62.

Colombo C, Cosentino M, Marino F, Rasini E, Ossola M, Blandini F, Mangiagalli A, Samuele A, Ferrari M, Bombelli R, Lecchini S, Nappi G, Frigo G. Dopaminergic Modulation of Apoptosis in Human

Peripheral Blood Mononuclear Cells. Possible Relevance for Parkinson's Disease. Ann N Y Acad Sci 2003;1010:679–682. [PubMed: 15033811]

Cueva JP, Giorgioni G, Grubbs RA, Chemel BR, Watts VJ, Nichols DE. trans-2,3-dihydroxy-6a,7,8,12b-tetrahydro-6H-chromeno[3,4-c]isoquinoline: synthesis, resolution, and preliminary pharmacological characterization of a new dopamine D1 receptor full agonist. J Med Chem 2006;49:6848–6857. [PubMed: 17154515]

Davidson M, Harvey PD, Bergman RL, Powchik P, Kaminsky R, Losonczy MF, Davis KL. Effects of the D-1 agonist SKF-38393 combined with haloperidol in schizophrenic patients. Arch Gen Psychiatry 1990;47:190–191. [PubMed: 2405808]

Dawson TM, Dawson VL. Molecular pathways of neurodegeneration in Parkinson's disease. Science 2003;302:819–822. [PubMed: 14593166]

Desai RI, Terry P, Katz JL. A comparison of the locomotor stimulant effects of D1-like receptor agonists in mice. Pharmacol Biochem Behav 2005;81:843–848. [PubMed: 16000217]

Docherty JR. Subtypes of functional alpha1- and alpha2-adrenoceptors. Eur J Pharmacol 1998;361:1–15. [PubMed: 9851536]

Fancellu R, Armentero MT, Nappi G, Blandini F. Neuroprotective effects mediated by dopamine receptor agonists against malonate-induced lesion in the rat striatum. Neurol Sci 2003;24:180–181. [PubMed: 14598076]

Gilmore JH, Watts VJ, Lawler CP, Noll EP, Nichols DE, Mailman RB. "Full" dopamine D1 agonists in human caudate: biochemical properties and therapeutic implications. Neuropharmacol 1995;34:481–488.

Goulet M, Madras BK. D1 dopamine receptor agonists are more effective in alleviating advanced than mild parkinsonism in 1-methyl-4-phenyl-1,2,3, 6-tetrahydropyridine-treated monkeys. J Pharmacol Exp Ther 2000;292:714–724. [PubMed: 10640310]

Huang X, Lawler CP, Lewis MM, Nichols DE, Mailman RB. D1 dopamine receptors. Int Rev Neurobiol 2001;48:65–139. [PubMed: 11526741]

Hurley MJ, Jenner P. What has been learnt from study of dopamine receptors in Parkinson's disease? Pharmacol Ther 2006;111:715–728. [PubMed: 16458973]

Hyttel J, Bøgesø KP, Perregaard J, Sánchez C. The pharmacological effect of citalopram resides in the (S)-(+)-enantiomer. J Neur Trans 1992;V88:157–160.

Izake EL. Chiral discrimination and enantioselective analysis of drugs: an overview. J Pharm Sci 2007;96:1659–1676. [PubMed: 17221858]

Jenner P. Dopamine agonists, receptor selectivity and dyskinesia induction in Parkinson's disease. Curr Opin Neurol 2003;16(Suppl 1):S3–7. [PubMed: 15180131]

Johnson DE, Ochieng J, Evans SL. The growth inhibitory properties of a dopamine agonist (SKF 38393) on MCF-7 cells. Anticancer Drugs 1995;6:471–474. [PubMed: 7670147]

Lewis MM, Huang X, Nichols DE, Mailman RB. D1 and Functionally Selective Dopamine Agonists as Neuroprotective Agents in Parkinsons Disease. CNS Neurol Disord Drug Targets 2006;5:345–353. [PubMed: 16787233]

Mailman R, Huang X, Nichols DE. Parkinson's disease and D1 dopamine receptors. Curr Opin Investig Drugs 2001;2:1582–1591.

Mottola DM, Brewster WK, Cook LL, Nichols DE, Mailman RB. Dihydrexidine, a novel full efficacy D1 dopamine receptor agonist. J Pharmacol Exp Ther 1992;262:383–393. [PubMed: 1352553]

Mu Q, Johnson K, Morgan PS, Grenesko EL, Molnar CE, Anderson B, Nahas Z, Kozel FA, Kose S, Knable M, Fernandes P, Nichols DE, Mailman RB, George MS. A single 20 mg dose of the full D1 dopamine agonist dihydrexidine (DAR-0100) increases prefrontal perfusion in schizophrenia. Schizophr Res 2007;94:332–341. [PubMed: 17596915]

Muralikrishnan D, Ebadi M. SKF-38393, a dopamine receptor agonist, attenuates 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced neurotoxicity. Brain Res 2001;892:241–247. [PubMed: 11172770]

Neve KA, Seamans JK, Trantham-Davidson H. Dopamine receptor signaling. J Recept Signal Transduct Res 2004;24:165–205. [PubMed: 15521361]

Olanow CW, Agid Y, Mizuno Y, Albanese A, Bonuccelli U, Damier P, De Yebenes J, Gershanik O, Guttman M, Grandas F, Hallett M, Hornykiewicz O, Jenner P, Katzenschlager R, Langston WJ,

LeWitt P, Melamed E, Mena MA, Michel PP, Mytilineou C, Obeso JA, Poewe W, Quinn N, Raisman-Vozari R, Rajput AH, Rascol O, Sampaio C, Stocchi F. Levodopa in the treatment of Parkinson's disease: current controversies. Mov Disord 2004;19:997–1005. [PubMed: 15372588]

Pitfield SE, Bryant I, Penington DJ, Park G, Riese DJ 2nd. Phosphorylation of ErbB4 on tyrosine 1056 is critical for ErbB4 coupling to inhibition of colony formation by human mammary cell lines. Oncol Res 2006;16:179–193. [PubMed: 17120616]

Rascol O, Nutt JG, Blin O, Goetz CG, Trugman JM, Soubrouillard C, Carter JH, Currie LJ, Fabre N, Thalamas C, Giardina WW, Wright S. Induction by dopamine D1 receptor agonist ABT-431 of dyskinesia similar to levodopa in patients with Parkinson disease. Arch Neurol 2001;58:249–254. [PubMed: 11176963]

Schapira AH, Bezard E, Brotchie J, Calon F, Collingridge GL, Ferger B, Hengerer B, Hirsch E, Jenner P, Le Novere N, Obeso JA, Schwarzschild MA, Spampinato U, Davidai G. Novel pharmacological targets for the treatment of Parkinson's disease. Nat Rev Drug Discov 2006;5:845–854. [PubMed: 17016425]

Schneider JS, Sun ZQ, Roeltgen DP. Effects of dihydrexidine, a full dopamine D-1 receptor agonist, on delayed response performance in chronic low dose MPTP-treated monkeys. Brain Res 1994;663:140–144. [PubMed: 7850462]

Taylor JR, Lawrence MS, Redmond DE Jr, Elsworth JD, Roth RH, Nichols DE, Mailman RB. Dihydrexidine, a full dopamine D1 agonist, reduces MPTP-induced parkinsonism in monkeys. Eur J Pharmacol 1991;199:389–391. [PubMed: 1680717]

Temlett JA, Chong PN, Oertel WH, Jenner P, Marsden CD. The dopamine D1 partial agonist, CY208-243, exhibits antiparkinsonian activity in the MPTP-treated marmoset. Eur J Pharmacol 1988;156:197–206. [PubMed: 2977118]

Tsui JK, Wolters EC, Peppard RF, Calne RB. A double-blind, placebo controlled, dose ranging study to investigate the safety and efficacy of CY208-243 in patients with Parkinson's disease. Neurology 1989;39:56–58.

Villarreal JE, Guzman M, Smith CB. A comparison of the effects of d-amphetamine and morphine upon the locomotor activity of mice treated with drugs which alter brain catecholamine content. J Pharmacol Exp Ther 1973;187:1–7. [PubMed: 4746328]

Waldeck B. Three-dimensional pharmacology, a subject ranging from ignorance to overstatements. Pharmacol Toxicol 2003;93:203–210. [PubMed: 14629731]

Watts VJ, Lawler CP, Gonzales AJ, Zhou QY, Civelli O, Nichols DE, Mailman RB. Spare receptors and intrinsic activity: studies with D1 dopamine receptor agonists. Synapse 1995;21:177–187. [PubMed: 8584979]

Watts VJ, Neve KA. Sensitization of endogenous and recombinant adenylate cyclase by activation of D2 dopamine receptors. Mol Pharmacol 1996;50:966–976. [PubMed: 8863843]

Zhang J, Xiong B, Zhen X, Zhang A. Dopamine D_1 receptor ligands: where are we now and where are going. Med Res Rev. 200810.1002/med.20130

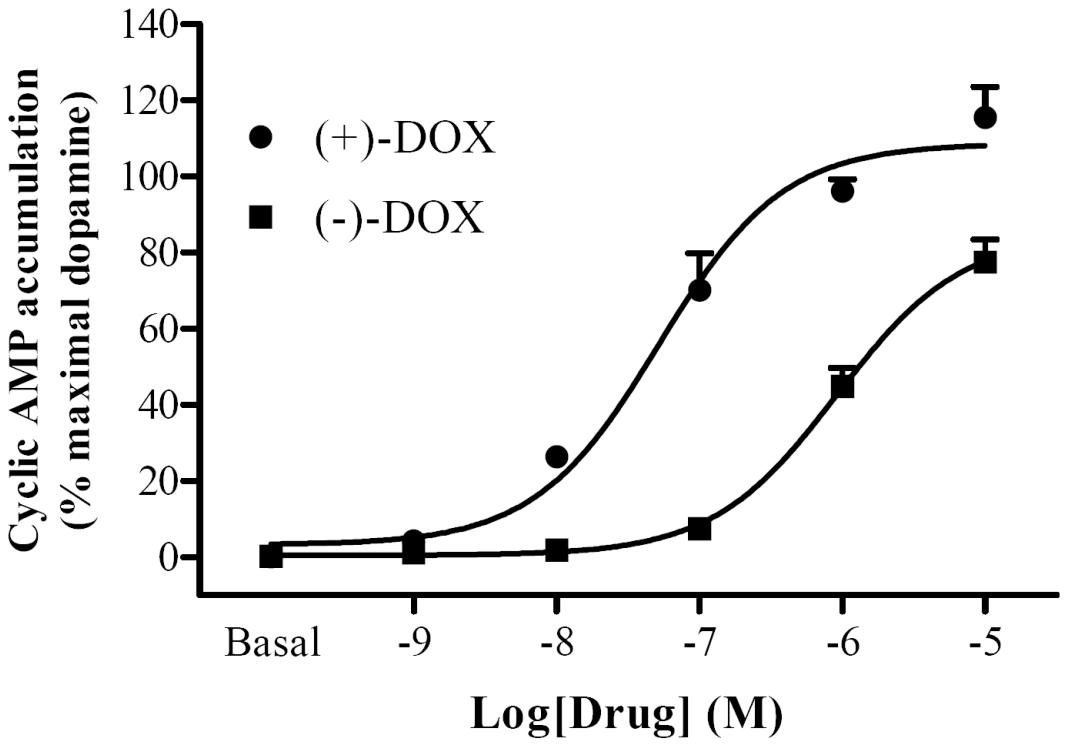

Figure 1. Dose-response curves for dopamine D_1 receptor-mediated stimulation of cyclic AMP accumulation

HEK-D_1 cells were incubated with increasing concentrations of dopamine, (+)-DOX, or (-)-DOX for 15 min at 37 °C. The data presented have been normalized to the maximal cyclic AMP accumulation observed in the presence of dopamine. Data shown are the mean ± SEM of six independent experiments assayed in duplicate.

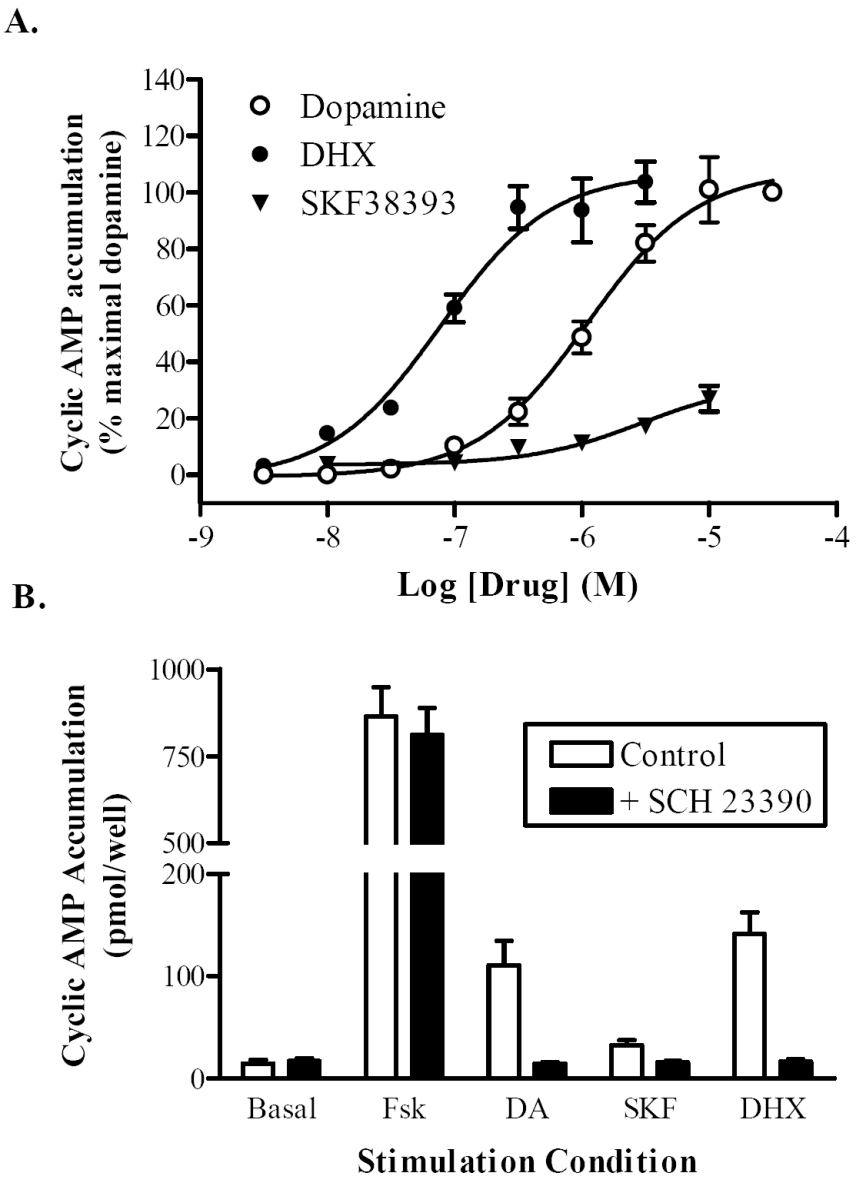

Figure 2. Characterization of a human D_1-like dopamine receptor in MCF7 cells

A. MCF7 cells were incubated with increasing concentrations of dopamine, DHX, or SKF38393 for 15 min at 37 °C. The data presented have been normalized to the maximal cyclic AMP accumulation observed in the presence of dopamine and are the mean ± SEM of three or four independent experiments assayed in duplicate. **B.** Cyclic AMP accumulation under basal conditions or following incubation with 3 μM forsklin (FSK), 5 μM dopamine (DA), 5 μM DHX, or 5 μM SKF38393 (SKF) in the absence (control) or presence of the D_1 dopamine receptor antagonist, SCH23390 (5 μM). The data presented are the mean ± SEM of three independent experiments assayed in duplicate.

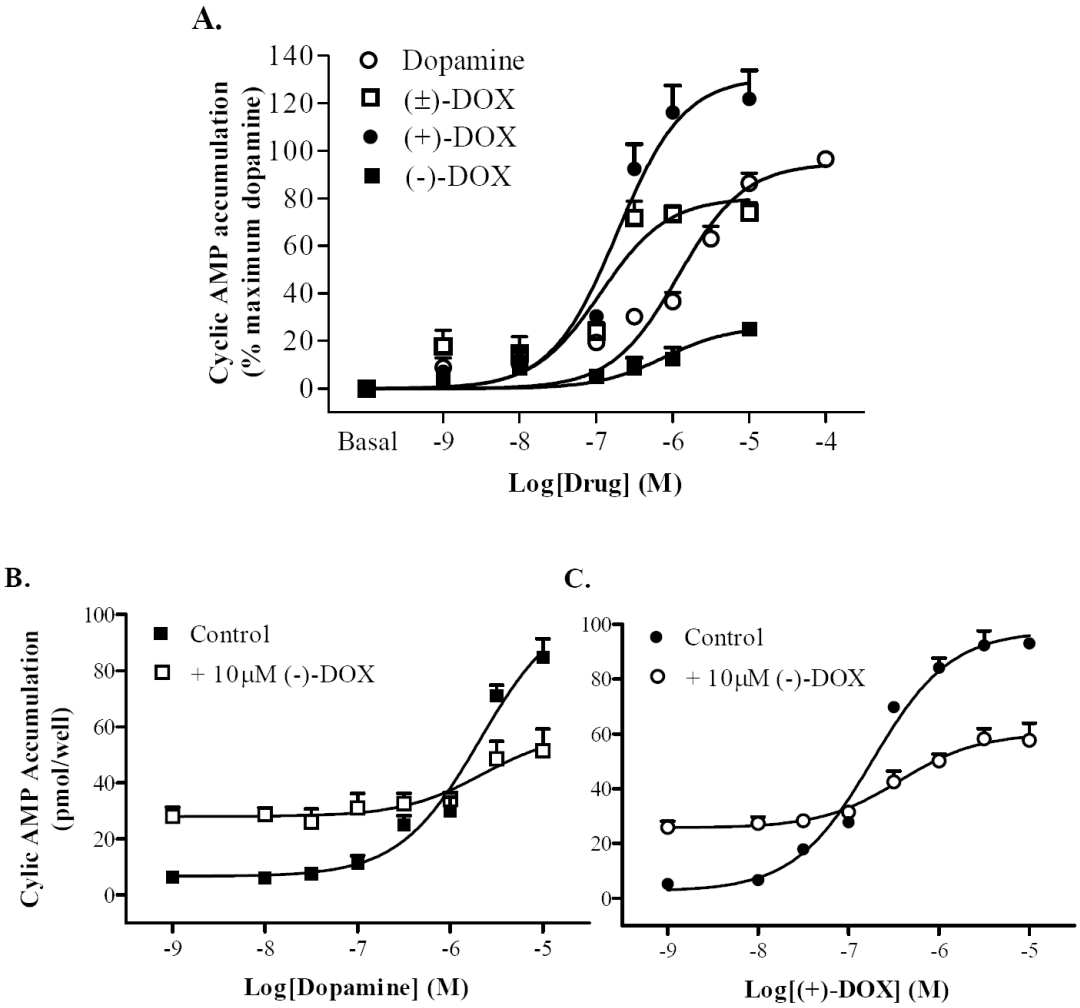

Figure 3. Dose-dependent stimulation of cyclic AMP at the endogenous dopamine D_1 receptor in MCF7 cells

A. MCF7 cells were incubated with increasing concentrations of dopamine, (±)-DOX, (+)-DOX, or (-)-DOX for 15 min at 37 °C. The data presented have been normalized to the maximal cyclic AMP accumulation observed in the presence of dopamine. **B., C.** Dose-response curves for dopamine- or (+)-DOX-stimulated cyclic AMP accumulation were completed in the absence (solid symbols) or the presence of 10 μM (-)-DOX (open symbols) as described above. The data presented in each figure are the mean ± SEM of three independent experiments assayed in duplicate.

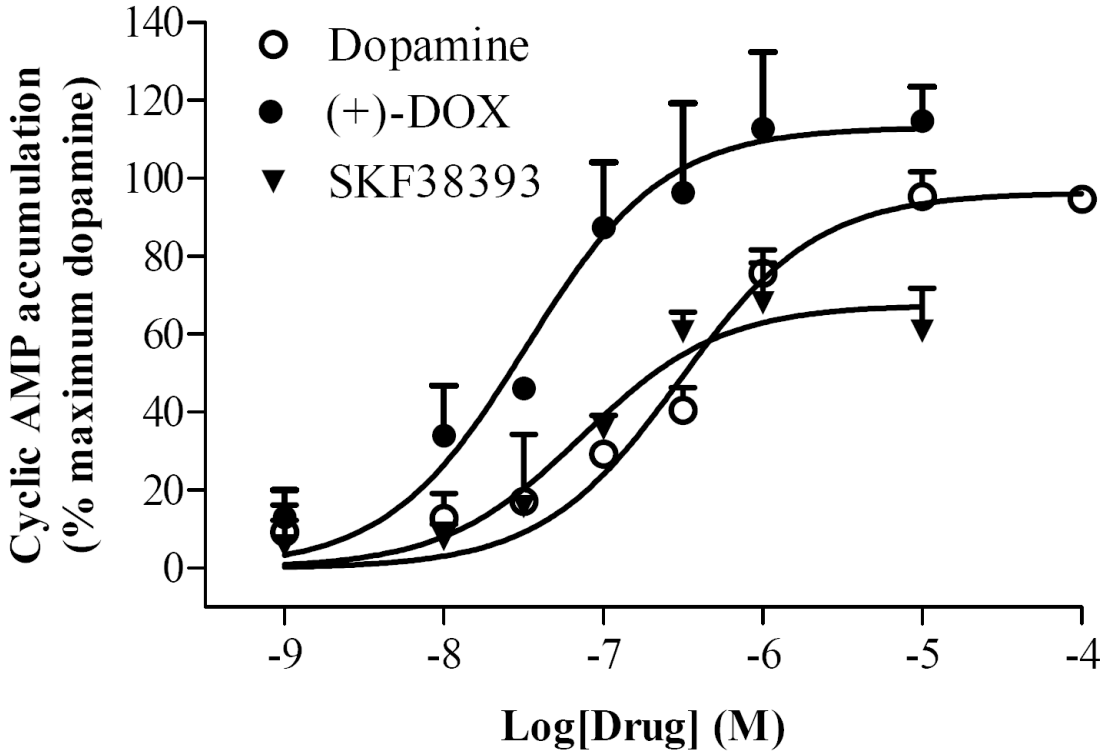

Figure 4. Dose-dependent stimulation of cyclic AMP in porcine striatal homogenate

Striatal tissue was incubated in the presence of increasing concentrations of dopamine, (+)-DOX, or SKF38393 for 15 min at 30 °C. The data presented have been normalized to the maximal cyclic AMP accumulation observed in the presence of dopamine and are the mean ± SEM of three independent experiments assayed in duplicate.

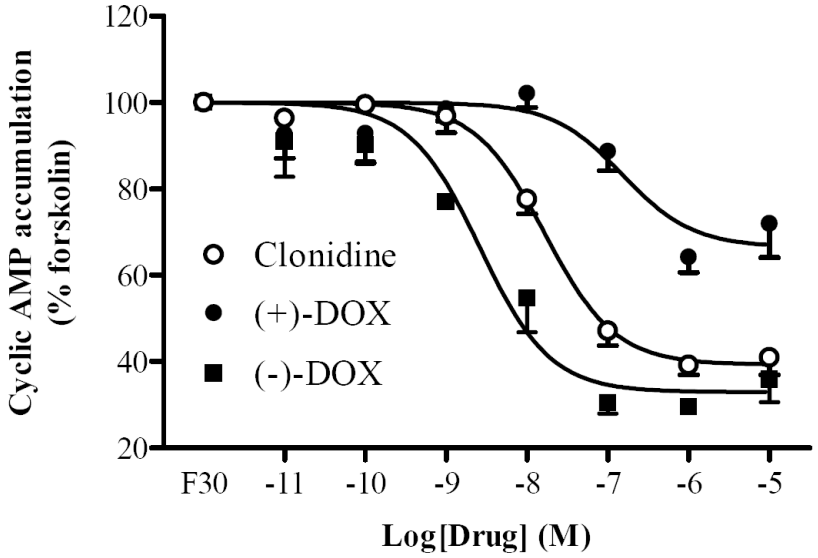

Figure 5. Dose-response curves for α_{2C} receptor-mediated inhibition of forskolin-stimulated cyclic AMP accumulation

HEK-α_{2C} cells were incubated with 30 μM forskolin in the presence of increasing concentrations of clonidine, (+)-DOX, or (-)-DOX for 15 min at 37 °C. The data presented have been normalized to the maximal cyclic AMP accumulation observed in the presence of forskolin alone and are the mean ± SEM of three independent experiments assayed in duplicate. The EC50 values for (-)-DOX and clonidine in these experiments were 4.4 ± 2.3 nM and 17 ± 3.2 nM, respectively.

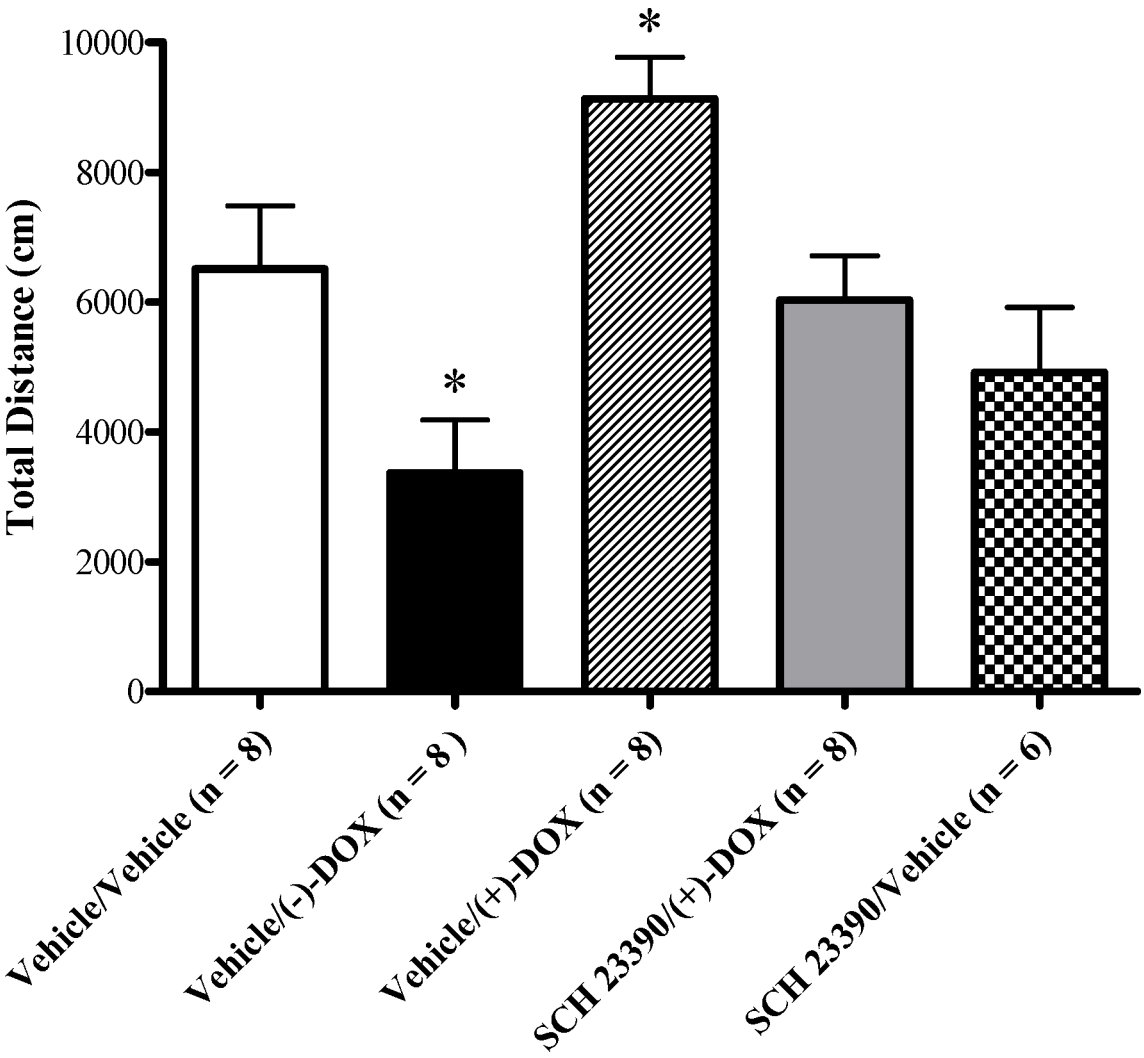

Figure 6. Effects of enantiomers of DOX on locomotor activity

Swiss-Webster mice were administered either vehicle/vehicle, vehicle/5.0 mg/kg (-)-DOX, vehicle/5.0 mg/kg (+)-DOX, 0.03 mg/kg SCH23390/5.0 mg/kg (+)-DOX, or 0.03 mg/kg SCH23390/vehicle. Data depicted are total distance traveled (cm) during the first 30 min of the test session. Student-Newman-Keuls post-hoc analysis: * $p < 0.05$ compared to vehicle/vehicle group.