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Typical antipsychotics exhibit inverse agonist activity at rat dopamine D1-like receptors expressed in Sf9 cells

Michael W. Martin, Allelia W. Scott, Douglas E. Johnston Jr., Suzy Griffin, Robert R. Luedtke *

Department of Pharmacology and Neuroscience, University of North Texas Health Science Center at Fort Worth, 3500 Camp Bowie Boulevard, Fort Worth, TX 76107, USA

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Abstract

The baculovirus system has been used to express the rat dopamine D1 receptors in *Spodoptera frugiperda* (Sf9) cells. A panel of typical antipsychotics including, α-flupenthixol, fluphenazine and thioridizine were found to inhibit dopamine-dependent stimulation of adenylyl cyclase. However, these compounds were also found to inhibit adenylyl cyclase activity in the absence of agonist in Sf9 cells expressing dopamine D1-like receptors. Therefore, these nonselective dopamine receptor compounds displayed negative intrinsic or inverse agonist activity. None of the compounds tested were neutral antagonists. © 2001 Published by Elsevier Science B.V.

Keywords: Adenylyl cyclase stimulation; Antipyschotic; Baculovirus expression; Dopamine D1 receptor; Dopamine receptor; Inverse agonist

1. Introduction

There are two major pharmacologic classes of dopamine receptors, D1-like and D2-like receptors (Lachowicz and Sibley, 1997). Agonist stimulation of the dopamine D1-like receptors (D1 and D5 receptors) causes an increase in adenylyl cyclase activity (Tiberi et al., 1991; Tiberi and Caron, 1994), stimulates K^+ efflux (Laitinen, 1993) and reduces N- and P-type Ca^{2+} currents (Surmeier et al., 1995). Agonist activation of the dopamine D2-like receptors (D2, D3 and D4) inhibits adenylyl cyclase, stimulates arachadonic release (Chio et al., 1994a,b), increases phosphatidylinositol hydrolysis, enhances Ca^{2+} mobilization (Vallar et al., 1990) and regulates K^+ channels (Liu et al., 1996).

There are multiple neurological and neuropsychiatric disorders that appear to involve disturbances of dopamine neurotransmission, including Parkinson's disease, Tourette's syndrome, tardive dyskinesia, schizophrenia and schizoaffective disorders (Nestler, 1994). Dopamine receptor activation has been implicated in vision (Djamgoz and Wagner, 1992), renal vasodilator and sodium excretion, (Aperia, 1994) and thermoregulation (Salmi, 1998). Studies in non-human primates and humans have implicated the dopamine D1-like receptors located in the prefrontal cortex in working memory (Williams and Goldman-Rakic, 1996).

In addition, studies on the molecular mechanisms of the reward system associated with drugs of abuse have shown that agonists at dopamine D1-like receptors can substitute for psychostimulants in self-administered behavioral paradigms (Self and Stein, 1992). Antagonists at both dopamine D1-like and D2-like receptors have been found to decrease the reinforcing effects of cocaine in both rats and monkeys (Woolverton and Virus, 1989; Bergman et al., 1990; Ward et al., 1996). Furthermore, gene "knockout" studies in mice have implicated dopamine D1-like receptors in cocaine-dependent hypermotor activity (Xu et al., 1994). Therefore, understanding the mechanisms associated with dopamine receptor subtype expression and regulation has important clinical ramifications.

Although dopamine D1-like receptor selective antagonists do not appear to have antipsychotic activity, the role of dopamine D1-like receptors in neuropsychiatric illness is suggested by the observation of a worsening of symptoms in some patients with schizophrenia following administration of a dopamine D1-like selective antagonist (Karlsson et al., 1995). Furthermore, studies by Swartz et al. suggest that developmental expression of the dopamine D3 receptor may be dependent upon dopamine D1-like recep-

* Corresponding author. Tel.: +1-817-735-2611; fax: +1-817-735-2091.

E-mail address: rluedtke@hsc.unt.edu (R.R. Luedtke).

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tor tone. Stimulation of co-expressed dopamine D1 and D3 receptors can lead to opposite effects, which is consistent with the opposite effect of the dopamine D1 and D3 receptors on cAMP formation. However, stimulation of dopamine D1 and D3 receptors in the shell of the nucleus accumbens results in synergistic effects. Several pieces of evidence point to the dopamine D3 receptor subtype as being pivotal in the clinical efficacy of both typical and atypical antipsychotics, while alterations in the expression and/or sensitivity of dopamine D2 receptors have been implicated in the irreversible motor dysfunction associated with typical antipsychotics (Sokoloff et al., 1990; Schwartz et al., 1998).

A hypothesis of an opposing interaction between dopamine D1-like and D2-like receptors in neuropsychiatric illness is supported by the studies of Goldman-Rakic et al. who found a 30% to 60% downregulation of dopamine D1 and D5 receptor mRNA in the prefrontal cortex of rhesus monkeys chronically treated with various antipsychotic drugs (Lidow and Goldman-Rakic, 1994; Lidow et al., 1997). Surprisingly, although cortical dopamine D1-like receptors and mRNA were found to be downregulated, there appeared to be no change in dopamine D1-like receptors expressed in the caudate. These authors hypothesized that modulation of cortical dopamine D1-like receptor expression following administration of antipsychotics might be an important component of the therapeutic utility of antipsychotic drugs.

We have used the baculovirus system to express the rat dopamine D1 and D5 receptors in *Spodoptera frugiperda* (Sf9) cells (Summers and Smith, 1987). The typical antipsychotics, flupenthixol, fluphenazine and thioridazine, inhibit both basal and dopamine-dependent stimulation of adenylyl cyclase. Therefore, these compounds were found to have negative intrinsic activity at dopamine D1-like receptors.

2. Materials and methods

2.1. Materials

DNA clones for the rat dopamine D1 and D5 receptors were generously provided by Dr. Marc Caron of Duke University (Tiberi et al., 1991). Haloperidol, (+)-butaclamol, α-*cis*-flupenthixol, fluphenazine, thioridazine, and 3-isobutylmethylxanthine were from Research Biochemical (Natick, MA).

2.2. Cell culture and construction of recombinant viral stocks

The coding region (1373 bp) for the dopamine D1 receptor was excised at *Bam*HI sites, purified by agarose electrophoresis and subcloned into the pVL1392 vector (Invitrogen). The orientation of the insert was verified by digesting the plasmid with *Eco*RI, which is a unique restriction site within the clone and the pGEM polylinker. The coding region (1400 bp) for the rat dopamine D5 receptor was excised by digesting with *Eco*RI and *Xba*I, purified and the insert was ligated into pVL1392. Subclones were verified by digestion using these enzymes.

The pVL1392 plasmids containing genes coding for the rat dopamine D1 or D5 receptors were used to obtain recombinant baculovirus stocks capable of directing the synthesis of the receptors in Sf9 cells (American Type Culture Collection, CRL 1711). Sf9 cells were grown in TNM-FH medium (complete Graces insect medium supplemented with yeastolate, lactalbumin hydrolysate, and 10% HI-FBS) at 27–28°C (Summers and Smith, 1987). Monolayers of cells were transfected with each of the pVL1392-receptor gene constructs (500 ng DNA) mixed with 100 ng of linearized BacPak6 (*Autographica californica* nuclear polyhedrosis virus; AcMNPV) viral DNA (Clontech) and 5 μg Lipofectin reagent (Gibco/BRL) using a transfection protocol (Summers and Smith, 1987). A plaque assay was used to select recombinant virus. Radioligand binding of [^{3}H]SCH 23390 to cell membrane preparations was used to identify recombinant viral stocks capable of directing the expression of dopamine D1-like receptors. Viral stocks were propagated in suspension cultures of Sf9 cells, tittered by a plaque assay, and stored in the dark at 4°C (Luedtke et al., 1999).

2.3. Membrane preparation

Sf9 cell was harvested from suspension cultures by centrifugation at $6000 \times g$ for 10 min. The cell pellet was resuspended in cold (4°C) homogenization buffer (50 mM NaHEPES, pH 7.4, 0.1 mM EDTA, 1 mM dithiothreitol) by vigorous vortexing and then homogenized with a Polytron (Brinkmann Instruments, Westbury, NY). The homogenate was centrifuged at $40{,}000 \times g$ for 10 min, 4°C and the resulting crude membrane pellet was resuspended in homogenization buffer by polytron and centrifuged. For adenylyl cyclase assays, the resulting washed membrane pellet was resuspended in homogenization buffer containing 5 mM $MgCl_2$ (approximately 1 mg protein/ml) and assayed immediately. For ligand binding assays, membrane pellets were resuspended in 50 mM Tris–HCl/150 mM NaCl/1.0 mM EDTA buffer, pH 7.5 (0.1–0.5 mg protein/ml) immediately prior to assay.

2.4. Radioligand binding assays

Membranes were diluted with 50 mM Tris–HCl/ 150 mM NaCl/1 mM EDTA, pH 7.5 buffer (5–25 μg protein/ 50 μl) and incubated in a total volume of 150 μl with [^{3}H]SCH 23390 (Dupont-NEN) at 37°C for 60 min. The concentration of [^{3}H]SCH 23390 ranged from 100 pM to 10 nM for direct radioligand binding experiments and from 0.5 to 1 nM for competitive displacement experi-

ments. Nonspecific binding was defined as filter-bound radioactivity measured in the presence of 10 μM (+)-butaclamol. The reactions were terminated by the addition of cold wash buffer (10 mM Tris–HCl, 150 mM NaCl, pH 7.5 at 4°C) and rapid filtration over glass fiber filters (Schleicher and Schuell No. 30). Filters were washed with 10 ml of wash buffer and the bound radioactivity quantitated at a counting efficiency of 50% after overnight incubation of samples in scintillation fluid. The protein concentration of the membranes was determined using a BCA reagent (Pierce) and bovine serum albumin as the protein standard.

Estimates of the equilibrium dissociation constant (K_d) and maximum number of binding sites (B_{max}) were obtained using unweighted nonlinear regression analysis of data modeled according to the equation describing mass action binding to one or more independent populations of binding sites (n)

$$[B] = \sum_{i=1}^{n} \left(B_{\max_i} \times [L] \right) / \left([L] + K_{di} \right)$$

where $[B]$ is the concentration of radioligand bound at any given radioligand concentration $[L]$ (McGonigle et al., 1984). Nonlinear fitting procedures utilize Jandel Scientific Tablecurve™ software and a nonlinear fitting algorithm (Magar, 1972). Initial estimates of K_d and B_{max} were obtained using unweighted linear regression analysis of data transformed by the method of Scatchard (1949).

Data from competitive inhibition experiments were modeled using nonlinear regression analysis (Magar, 1972) to determine the concentration of inhibitor that inhibits 50% of the specific binding of the radioligand (IC_{50} value). The competition curves were modeled for a single site using the following equation:

$$B = \frac{B_0}{1 + (L/IC_{50})} + B_{ns}$$

where B is the amount of ligand bound to tissue, B_0 is the amount of ligand bound in the absence of competitive inhibitor, L is the concentration of the competitive inhibitor, B_{ns} is the nonspecific binding of the radioligand (defined using a high concentration of a structurally dissimilar competitive inhibitor) and IC_{50} is the concentration of competitive inhibitor that inhibits 50% of the total specific binding. The values for B_{ns} and B_0 were constrained using experimentally derived values.

2.5. Adenylyl cyclase assay

Adenylyl cyclase activity was determined by the method of Salomon et al. (1974). Assays were initiated by the addition of 20 μl of membranes (20–50 μg protein) to a solution containing the following reagents (final concentrations) in an assay volume of 100 μl: 50 mM HEPES (pH 7.4), 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, 0.1 mM EGTA, 0.1 mM 3-isobutylmethylxanthine, 0.05 mM cyclic AMP, 0.1 mM ATP, 2.0×10^6 cpm of [^{32}P]-ATP, 0–100 μM GTP, 50 units/ml creatine phosphokinase and 5 mM phosphocreatine. Dopamine and water-soluble agonists and antagonists were dissolved in 0.1% (w/v) ascorbic acid. Water-insoluble drugs were dissolved in N,N'-dimethylformamide (DMF) or dimethylsulfoxide (DMSO). Experiments were performed to verify that the amount of solvent had no effect on adenylyl cyclase assay. Under standard conditions, the production of cyclic AMP was linear for at least 30 min. Reactions were terminated by the addition of 100 μl of a solution containing 2% SDS, 4.5 mM ATP, 1 mM cyclic AMP and 50 mM Tris (pH 7.5) with [^{3}H]cyclic AMP (50 μl, 10,000 cpm). Membranes were solubilized in a boiling water bath for 3 min and the contents of each tube were applied to a Dowex AG50 W-X4 (Bio Rad, Richmond, CA; 200–400 mesh, H^+ form) cation exchange resin. The remaining ATP and cyclic AMP were eluted onto neutral alumina columns (ICN Nutritional Biochemical, Cleveland, OH) with H_2O. The cyclic AMP was then eluted with 100 mM imidazole–HCl buffer, pH 8.0. The eluate was dissolved in 10 ml of scintillation fluid (Ecolume, ICN) with a counting efficiency of approximately 35% for tritium and 80% for [^{32}P]. Recoveries of [^{3}H]cAMP were approximately 70% and [^{32}P]-cAMP accumulation was corrected for recovery.

The dose–response curves of adenylyl cyclase activity were fit to a four-parameter logistic equation (DeLean et al., 1980) by nonlinear least-squares regression analysis according to the method of Marquardt and Levenberg (Magar, 1972) using the following equation:

$$Y = \frac{(a - d)}{1 + (x/c)^b} + d$$

In this equation, Y is the level of enzyme activity, d is the maximal level of enzyme activity, x is the concentration of agonist and c is the concentration of agonist that causes a half-maximal effect (EC_{50}), a is the enzyme activity in the absence of agonist (basal) and b is the slope of the curve. Curve fitting was done using Jandel Scientific Tablecurve™ software. The EC_{50} values obtained from dose–response curves measured, in the absence and presence of increasing concentrations of antagonists, were used to construct Schild plots (Arunlakshana and Schild, 1959). The data were fit to the equation:

$$\log(A'/A - 1) = -\log K_i + \log B$$

In this equation, A' and A are the EC_{50} values determined in the presence and absence of antagonist, respectively, K_i is the equilibrium dissociation constant of the antagonist-receptor complex and B is the concentration of antagonist. Estimates of K_i were obtained using unweighted linear regression analysis of the transformed data.

3. Results

3.1. Expression and pharmacologic characterization of dopamine D1 and D5 receptors in Sf9 cells using the baculovirus expression system

The inserts from genomic clones coding for the rat dopamine D1 (in pGEM 3Z) and D5 (in pGEM 4Z) receptors (Tiberi et al., 1991; Tiberi and Caron, 1994) were subcloned into the pVL vector for the preparation of recombinant baculoviruses capable of directing the expression of either rat dopamine D1 or D5 receptors in Sf9 cells. Direct radioligand binding studies indicated that [^{3}H]SCH 23390 bound to the rat dopamine D1 and D5 receptors expressed in Sf9 cells with high affinity (0.8 ± 0.1 and 2.5 ± 0.1 nM for dopamine D1 and D5 receptors, respectively). The level of expression of rat dopamine D1 and D5 receptors in Sf9 cells can be varied by varying either the multiplicity of infection and/or the post-infection time at which the infected Sf9 cells are harvested (data not shown). Competitive radioligand binding studies confirm that the pharmacologic properties of rat dopamine D1 and D5 receptors are similar (Table 1). Fluphenazine and α-flupenthixol, which are nonselective antagonists at dopamine D1 and D2 receptors, were potent inhibitors of [^{3}H]SCH 23390 binding. Stereoselectivity of the binding of (+)-butaclamol and (−)-butaclamol isomers was observed. Low potency of inhibition was observed for dopamine D2-like receptor selective antagonists haloperidol, sulpiride and eticlopride, as well as the serotonin receptor antagonist ketanserin (data not shown). As noted in previous studies, dopamine receptor antagonists tend to have slightly higher affinity at dopamine D1 receptors (Table 1) but the selectivity for dopamine D1 verses D5 receptor subtypes only ranges from 1- to 10-fold (Sunahara et al., 1991; Tiberi and Caron, 1994; Luedtke et al., 1999).

Table 1
Comparison of the affinities of compounds for rat dopamine D1-like receptors expressed in Sf9 cells

Compound	Dissociation constants (nM)		Ratio D1/D5
	D1 receptor	D5 receptor	
SCH 23390[a]	0.8 ± 0.1	2.5 ± 0.1	0.32
α-Flupenthixol	0.7 ± 0.4	6.6 ± 2.8	0.11
SCH 23390	1.1 ± 0.3	6.6 ± 0.7	0.17
(+)-Butaclamol	2.3 ± 0.7	9.3 ± 1.2	0.25
Fluphenazine	2.3 ± 1.0	25 ± 6.7	0.10
Thiothixene	37 ± 14	118 ± 103	0.31
Thioridizine	64 ± 25	397 ± 178	0.16
Haloperidol	157 ± 65	407 ± 21	0.39
(−)-Butaclamol	> 10,000	> 10,000	–

The baculovirus expression system was used to direct the expression of dopamine receptors in Sf9 cells. [^{3}H]SCH 23390 was used to label dopamine receptors.

[a] Dissociation constants obtained using a Scatchard analysis of direct radioligand binding experiments (K_d values) were obtained using [^{3}H]SCH 23390 and were used for the calculation of the equilibrium dissociation constants for the competitive inhibitors (K_i values) using the equation of Cheng and Prussoff (1973). Mean K_i values were calculated using data from at least three competitive radioligand binding experiments ± the standard error of the mean.

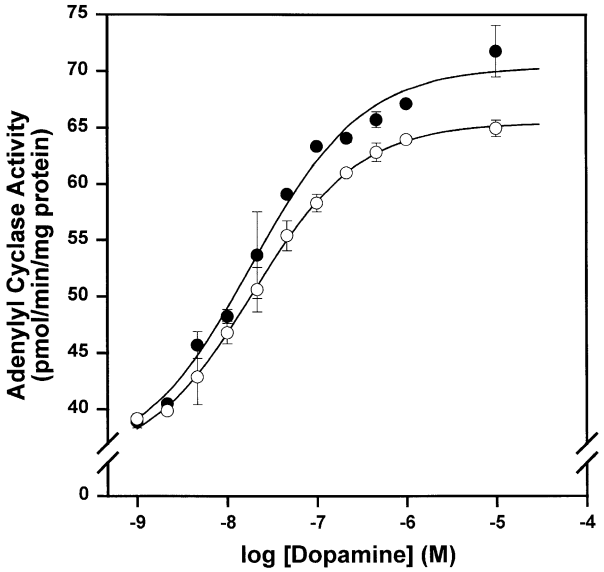

Fig. 1. Agonist stimulation of adenylyl cyclase activity in Sf9 cell membranes expressing rat dopamine D1-like receptors. Dopamine-stimulated adenylyl cyclase activity in membranes from Sf9 cells expressing dopamine D1 (●) or D5 (○) receptors. Sf9 cells (50 ml, 5×10^5 cells/ml) were infected at multiplicity of infection = 2 with a recombinant baculovirus. Cells were harvested at 48 h and membranes were prepared. The receptor density was measured by [^{3}H]SCH 23390 binding assays (2.1 and 2.3 pmol/mg protein for D1 and D5, respectively). Adenylyl cyclase assays were performed in the presence of 100 μM GTP. Adenylyl cyclase activity (pmol/min/mg protein) was determined as a function of increasing concentration of dopamine. Each point represents the mean and range of duplicate determinations.

3.2. Modulation of adenylyl cyclase activity in Sf9 cells by dopamine D1-like receptors

Both rat dopamine D1 and D5 receptors appear to couple to endogenous Sf9 cell G protein since a dose-dependent stimulation of adenylyl cyclase activity by dopamine was observed. The EC_{50} values for dopamine-dependent adenylyl cyclase stimulation were in the micromolar range, which is consistent with previous studies on dopamine D1-like receptors (Fig. 1). Dopamine has no effect on adenylyl cyclase system in uninfected Sf9 cells. Generally, a 2- to 2.5-fold stimulation of adenylyl cyclase active above basal activity was observed with dopamine at a final concentration of 10^{-5} M (Fig. 1, Table 2). To further verify that the dopamine-dependent increase in adenylyl cyclase activity was mediated through the dopamine D1-like receptors, experiments were performed to determine if dopamine receptor antagonists could attenuate the observed dopamine-dependent stimulation of adenylyl cyclase. At a concentration of 10^{-6} M,

Table 2
Inhibition of dopamine-dependent stimulation of adenylyl cyclase in Sf9 cells expressing dopamine D1-like receptors

Treatment	Adenylyl cyclase activity (pmol/min/mg protein)	
	D1 receptor	D5 receptor
(1) Basal	18.5 ± 2.3	17.1 ± 3.4
(2) Dopamine (10 μM)	45.1 ± 3.8	40.3 ± 3.9
(3) Dopamine (10 μM) + Fluphenazine (1 μM)	23.7 ± 4.9	22.7 ± 3.5

Data shown are the means ± S.E.M. of five dopamine D1 receptor and three dopamine D5 receptor experiments with different Sf9 cell membrane preparations assayed in triplicate. The receptor density of these membrane preparations ranged from 1.8 to 4.9 pmol of receptor/mg protein.

fluphenazine decreased the effect of a maximally effective concentration of dopamine (10^{-5} M) on adenylyl cyclase stimulation (Table 2). SCH 23390 at a concentration of 10^{-6} M, only partially decreased dopamine dependent cyclase activity. Further studies demonstrated that SCH 23390 was a partial agonist at both dopamine D1 and D5 receptors expressed in Sf9 cells (data not shown).

3.3. Negative intrinsic activity of classic dopamine receptor ligands

A panel of dopamine receptor antagonists were tested for their effect on dopamine-dependent and basal adenylyl cyclase activity. Three drugs used clinically as typical antipsychotics were selected for study, α-flupenthixol, fluphenazine and thioridazine. Each of these compounds was found to decrease basal cyclase activity in Sf9 cells expressing either rat dopamine D1 or D5 receptors (Fig. 2). This decrease appeared to be receptor mediated because there was essentially no change in adenylyl cyclase activity when these compounds were tested for effect on the basal adenylyl cyclase activity using uninfected Sf9 cells. The mean change in basal adenylyl cyclase activity of uninfected Sf9 cells for two independent experiments using haloperidol, (+)-butaclamol, α-*cis*-flupenthixol, fluphenazine and thioridazine was within 5% of the mean basal value (Fig. 2). The mean decrease in adenylyl cyclase activity of Sf9 cells expressing either dopamine D1 ($n = 9$) or D5 ($n = 4$) receptors using haloperidol, (+)-butaclamol, α-*cis*-flupenthixol, fluphenazine and thioridazine was > 35% of the mean basal value (Fig. 2).

Each of these typical antipsychotics inhibited dopamine-dependent stimulation of adenylyl cyclase in a concentration-dependent manner and with a rank order of potency consistent with their affinity at dopamine D1-like receptors (Fig. 3, Table 1). All three of these antipsychotics appeared to have negative intrinsic activity at both rat dopamine D1 and D5 receptors expressed in Sf9 cells because at high concentrations dopamine-dependent adenylyl cyclase activity was inhibited below basal levels (Fig. 3).

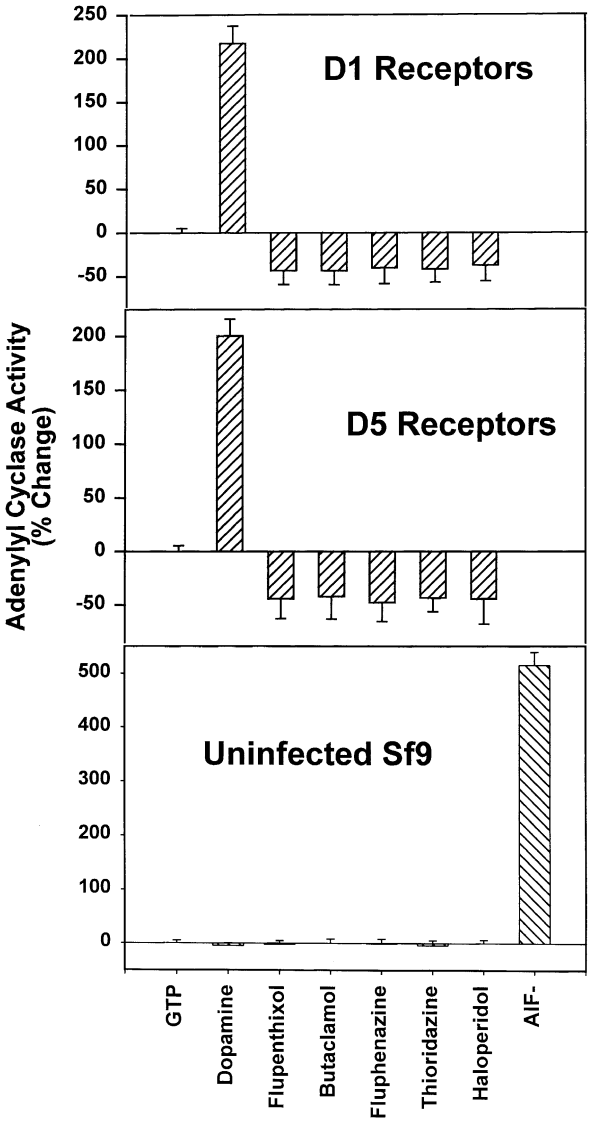

Fig. 2. The effect of dopamine and classical dopamine receptor ligands on adenylyl cyclase activity in Sf9 cell membranes expressing rat dopamine D1-like receptors. Sf9 cells (5×10^5 cells/ml) were infected with recombinant baculovirus containing the gene coding for the rat dopamine D1 (Top Panel) or the rat dopamine D5 (Middle panel) receptor at a multiplicity of infection equal to 2 for 48 h. Membrane preparations of the infected cells were assayed for adenylyl cyclase activity in the presence of dopamine or classical dopamine receptor antagonists. The data for the dopamine D1 receptor is the mean ± S.E.M. of nine independent experiments and the data for the dopamine D5 receptor is the mean ± S.E.M. of four independent experiments. Studies using uninfected Sf9 cells are also shown (Bottom panel) as the mean ± S.E.M. of the triplicates of two independent experiments. The adenylyl cyclase activity was corrected for protein concentration and plotted as the percent change in enzyme activity relative to samples which were incubated in the presence of 100 μM GTP but in the absence of drug (i.e., percent of basal activity). All of the drugs were tested at a final concentration of 10^{-6} M except dopamine, thioridazine and haloperidol, which were tested at 10^{-5} M. AlF_4 was included in the experiments using uninfected Sf9 cells as a positive control to verify the activity of adenylyl cyclase in membranes from uninfected Sf9 cells.

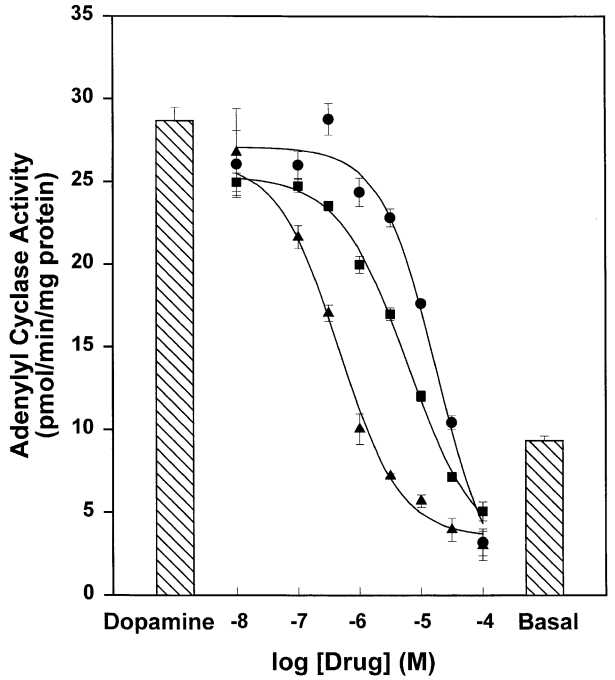

Fig. 3. Inhibition of dopamine-stimulated adenylyl cyclase activity by dopamine receptor compounds α-flupenthixol (▲), fluphenazine (■) and thioridazine (●) is shown. Sf9 cells were infected with the recombinant baculovirus capable of directing the expression of rat dopamine D1 receptors at a multiplicity of infection equal to 2 and cells were harvested 46 h after infection. Sf9 cell membranes were assayed in the presence of 100 μM GTP and in the absence (Basal) or presence of 10^{-5} M dopamine (Dopamine). Each sample contained GTP, dopamine and the indicated concentrations of competitive inhibitor. The mean and range of duplicate determinations are shown for one of three similar experiments.

Radioligand binding studies indicated that these drugs bind to dopamine D1-like receptors with high affinity. However, the apparent potency of these compounds for the inhibition of dopamine-dependent stimulation of adenylyl cyclase activity appeared to be less that of the estimated equilibrium dissociation constants (Fig. 3). This was likely due to the high concentrations of dopamine (10^{-5} M) required to achieve maximal stimulation of adenylyl cyclase activity. A more precise estimate of the potency of these compounds for dopamine D1-like receptors was obtained by a Schild analysis of their inhibitory effect on dopamine-stimulated adenylyl cyclase activity. Fig. 4 shows the concentration–response curves for the activation of adenylyl cyclase by dopamine in the absence and presence of increasing concentrations of α-flupenthixol (Fig. 4). As expected for competitive inhibition, a rightward shift of the concentration–response curve was observed as a function of increasing concentration of α-flupenthixol. Similar results were obtained using fluphenazine and thioridazine (data not shown). Consistent with the data presented in Figs. 2 and 3, the basal adenylyl cyclase activity also was observed to decrease with increased α-flupenthixol concentration (Fig. 4).

Schild analysis of the effect of α-flupenthixol, fluphenazine and thioridazine on dopamine-dependent stimulation of adenylyl cyclase activity in Sf9 cells expressing dopamine D1 receptors (Fig. 5) was used to estimate the affinity of these drugs at dopamine D1 receptors (Table 3). The dissociation constants (K_i values) obtained from the Schild analysis were in the same rank order of potency as the K_i values calculated from competitive radioligand binding studies using [^{3}H]SCH 23390 and Sf9 cell membranes expressing dopamine D1 receptors (Table 1).

We sought to investigate the dose–effect relationship between the negative intrinsic activities of α-flupenthixol, fluphenazine and thioridazine at dopamine D1-like receptors. Experiments were performed to determine the effect of these drugs on basal adenylyl cyclase activity as a function of inhibitor concentration (Fig. 6). Increasing concentration of these drugs resulted in a dose-dependent decrease of basal adenylyl cyclase activity. At the highest concentration tested (10^{-4} M), there was a 40% to 50% decrease in the observed basal activity. To maximize the concentration of the inhibitors, compounds were initially dissolved in *N*,*N*′-dimethylformamide. Solvent controls were performed to verify that the solvent in assay was not responsible for the observed decrease in basal activity. Even at the highest concentration of drug, residual basal activity was observed. The rank order of potency observed for these three antipsychotics was the same observed for

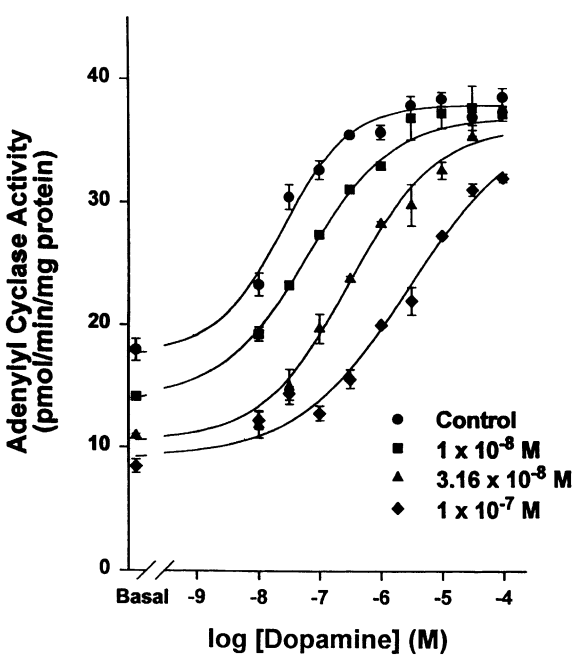

Fig. 4. Inhibition of dopamine-stimulated adenylyl cyclase activity of dopamine D1 receptors expressed in Sf9 cells by α-flupenthixol is shown. Sf9 cell membranes expressing dopamine D1 receptors were assayed for adenylyl cyclase activity with the indicated concentrations of dopamine and in the absence (●) or presence of 10 nM (■), 31.6 nM (▲), or 100 nM (♦) α-flupenthixol.

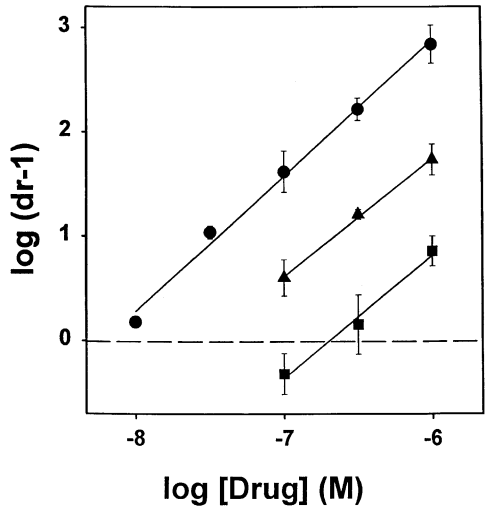

Fig. 5. Schild regression analysis of the inhibition of dopamine-dependent stimulation of dopamine D1 receptors expressed in Sf9 cells by flupenthixol, fluphenazine and thioridazine. Concentration–response curves for dopamine-dependent stimulation of adenylyl cyclase activity in membranes from Sf9 cells expressing rat dopamine D1 receptors were performed as shown in Fig. 4 at the indicated concentrations of α-flupenthixol (●), fluphenazine (▲) or thioridazine (■). The data is shown as Schild regression plots. Individual data points represent the mean ± S.E.M. for multiple independent experiments (α-flupenthixol, $n = 7$; fluphenazine, $n = 3$; thioridazine; $n = 3$).

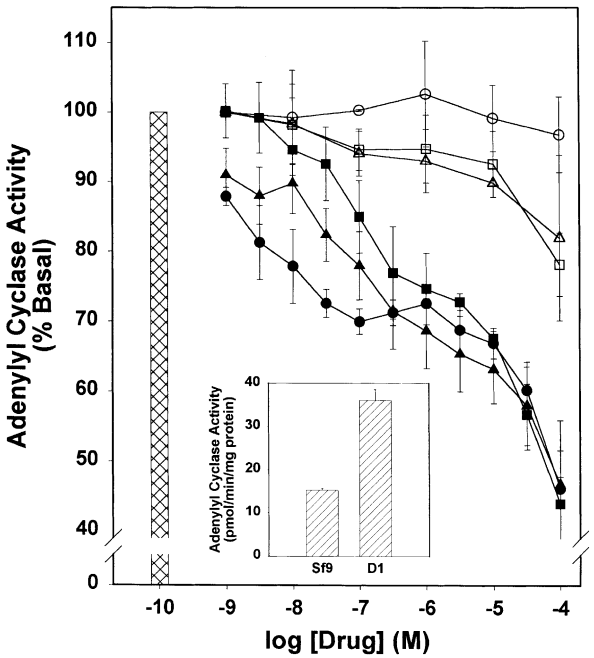

Fig. 6. Inhibition of basal adenylyl cyclase activity in uninfected Sf9 cells or Sf9 cells expressing rat dopamine D1 receptors by flupenthixol, fluphenazine and thioridazine. Adenylyl cyclase activity was measured using membranes from either uninfected Sf9 cells (open symbols) or Sf9 cells expressing rat dopamine D1 receptors (closed symbols). All assays were performed in the presence of 100 μM GTP with the indicated concentrations of compounds α-flupenthixol (●, ○), fluphenazine (▲, △) or thioridazine (■, □). The data has been normalized to show the percent inhibition of basal enzyme activity in each membrane preparation. Each point represents the mean ± S.E.M. of results from three independent experiments. The insert shows a comparison of the absolute basal adenylyl cyclase activity (pmol/min/mg protein) measured in uninfected Sf9 cell membranes (Sf9) and Sf9 cells membranes expressing the rat dopamine D1 receptor (D1).

the inhibition of dopamine-dependent adenylyl cyclase up to inhibitor concentration of approximately 10^{-7} to 10^{-6} M. However, at the higher concentrations of inhibitor the rank order of potency of the drugs is less clear. Since no clear plateau was observed at the high concentration portion of the dose–response curve, an ambiguity was introduced in determining the most appropriate estimation of an IC_{50} value for the inhibition of basal cyclase activity. The dose–response curves for the inhibition of basal adenylyl cyclase activity of membranes expressing dopamine D1 receptors (Fig. 6) appear visually to be biphasic. This is particularly true for the curve for flupenthixol.

Even though the Sf9 cells used in this study express a high level of dopamine D1 receptors, not all of the basal adenylyl cyclase activity would be due to constitutive dopamine D1-like receptor activation. Adenylyl cyclase activity measured in the presence of GTP was two- to threefold higher in membranes from Sf9 cells expressing either dopamine D1 or D5 receptors than the activity measured in uninfected cells (Fig. 6 insert). Therefore, the effect of the three antipsychotics on basal adenylyl cyclase of membranes from uninfected Sf9 cells was also examined. Essentially, no inhibition was observed in a dose range from 10^{-9} to 10^{-6} M. At higher concentration of the drugs, a 0% to 15% inhibition of enzyme activity was observed (Fig. 6). This data suggests that at low concentrations of drug, inhibition of basal activity is primarily receptor mediated, whereas at higher drug concentrations the mechanism of action of basal activity inhibition might be more complex. If 30% to 35% of the basal adenylyl cyclase activity were due to constitutive receptor activity, then the potency of the drugs tested would be in the nM range, which is consistent with an inhibition of this enzyme activity by a receptor mediated mechanism.

Table 3
The estimated affinity of typical antipsychotics at rat dopamine D1 receptors from Schild analysis
Data shown are the mean values for experiments conducted using dopamine D1 receptors expressed in different Sf9 cell membrane preparations. In this table, *n* represents the number of independent experiments. Each experimental data point was assayed in triplicate.

Compound	K_i (nM)	*n*
α-Flupenthixol	8	7
Fluphenazine	48	3
Thioridazine	200	3

4. Discussion

In this communication, we report the use the baculovirus expression system to express the rat dopamine D1

and the rat dopamine D5 receptors in Sf9 cells and to investigate the intrinsic activity of a panel of antipsychotics, including α-flupenthixol, fluphenazine and thioridazine. Rat dopamine D1-like receptors expressed in Sf9 cells retain their pharmacologic properties. These receptor subtypes appear to be coupled to endogenous Sf9 cell G protein since a dose-dependent stimulation of adenylyl cyclase activity can be achieved using dopamine. The ability to express dopamine D1-like receptors at a high receptor density in Sf9 cells has enabled us to study the effect of dopamine receptor compounds at D1-like receptors. The high receptor density increases the level of basal activity, thereby allowing us to identify compounds with negative intrinsic activity.

Studies from various laboratories have observed inhibition of basal second messenger systems by receptor antagonist selective for 5-HT_{2c}, β_2-adrenoceptor and δ-opioid receptors (Chidiac et al., 1994; Costa and Herz, 1989). Drugs possessing the ability to inhibit basal activity have been referred to as negative antagonists or inverse agonists. A modification of the original ternary complex model of G protein coupled receptors proposes a spontaneous, agonist-independent isomerization between the inactive (R) and the active (R^*) conformation of a receptor (Samana et al., 1993). Agonist binding would favor the stabilization of R^*, inverse agonist binding would favor stabilization of R and the binding of a neutral antagonist binding would not favor either conformation. This model predicts that in the absence of an agonist the total amount of R^* per cell would depend upon (1) a constant (J) describing the equilibrium between the agonist-independent active and inactive receptor conformations and (2) receptor density. Assuming J is independent of receptor density, the high levels of expression achieved using the baculovirus potentates the presence of both R and R^*. This model, therefore, predicts an increase in basal adenylyl cyclase activity as a function of receptor density for receptors coupled to the activation of adenylyl cyclase. This phenomenon has been documented for both β_2-adrenoceptor (Chidiac et al., 1994) and dopamine D1-like receptors (Tiberi and Caron, 1994; Cai et al., 1999). Since the binding of an inverse agonist would favor the R conformation, basal adenylyl cyclase would be predicted to decrease as a function of increased inverse agonist concentration.

For the system described in this report, the antipsychotics fluphenazine, α-*cis*-flupenthixol, thioridazine and haloperidol were found to be inverse agonists at both rat dopamine D1 and D5 receptors expressed in Sf9 cells (Fig. 2). The observed inhibition of basal adenylyl cyclase activity by fluphenazine, (+)-butaclamol, α-*cis*-flupenthixol, thioridazine and haloperidol suggests that both dopamine D1 and D5 receptors expressed in Sf9 cells assume the R^* active conformation in the absence of agonist. This active receptor conformation appears to be destabilized when the neurotransmitter binding site is occupied with an antipsychotic drug. There are several observations in this report which support the hypothesis that a portion of basal adenylyl cyclase activity is mediated by dopamine D1-like receptors. First, a decrease in basal adenylyl cyclase activity was not observed when uninfected Sf9 cells were evaluated. Second, the adenylyl cyclase basal activity is greater in Sf9 cells expressing dopamine D1-like receptors than in uninfected Sf9 cells (Fig. 6 insert). If there was no R^* conformation, then the basal activity of infected and uninfected Sf9 cells should be essentially the same. Finally, the high potency and the rank order of potency observed for the inhibition of basal adenylyl cyclase with fluphenazine, α-*cis*-flupenthixol and thioridazine (Fig. 6), seen at the lower drug concentrations (10^{-9} to 10^{-6} M), is consistent with the pharmacologic properties of dopamine D1-like receptors.

The rank order of potency for the antipsychotics fluphenazine, α-*cis*-flupenthixol and thioridazine appeared to be lost at the high-dose portions (10^{-6} to 10^{-4} M) of the basal adenylyl cyclase inhibition curves shown in Fig. 6. At these high concentrations, the inhibition of adenylyl cyclase activity may not be receptor mediated. When similar studies were performed using membranes from Sf9 cell lacking the dopamine D1 receptor, significant inhibition of basal adenylyl cyclase activity was also observed, but only at the higher drug concentrations (10^{-6} to 10^{-4} M). This data suggests that at high concentrations, these drugs inhibit enzyme activity in a receptor-independent manner. This non-receptor mediated inhibition of basal activity at the high concentrations of drug is likely responsible for the biphasic nature of the inhibition curves (Fig. 6). However, if it is assumed that the initial portion of the basal inhibition curves is receptor mediated (30% to 35% inhibition), then the IC_{50} values for these three antipsychotics would be in a nanomolar range. The rank order of these values would be consistent with K_i values obtained from both radioligand binding studies and Schild analysis. Therefore, the inhibition of basal activity observed at drug concentrations ranging from 10^{-9} to 10^{-6} M would be consistent with a receptor mediated phenomenon, where the R conformation is stabilized by occupation of the binding site with the inverse agonist.

Our studies are consistent with the work of Cai et al. (1999) who expressed rat dopamine D1 receptors in PC2 cells. They also observed inverse agonist activity of classic dopamine receptor antagonists at D1 receptors. In their studies, SCH 23390 was found to be a neutral antagonist, whereas we often found that SCH 23390 was a partial agonist (data not shown). In addition, we have documented that while at low concentrations several of the compounds that we tested appeared to inhibit adenylyl cyclase in a receptor-dependent manner, at higher concentrations the attenuation of adenylyl cyclase activity appeared to be receptor independent.

The studies presented in this communication present evidence that antipsychotics are not simply neutral antagonists at dopamine receptors. On the contrary, they have

intrinsic activity by virtue of their ability to attenuate an agonist-independent, receptor-mediated, G protein-linked second messenger signal. The intrinsic activity of antipsychotics at dopamine D1-like receptors, coupled with their ability to regulate the expression of dopamine D1-like receptors in the cortex of primates, may contribute to their clinical efficacy in the treatment of neuropsychiatric illnesses.

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References

Aperia, A., 1994. Dopamine action and metabolism in the kidney. Curr. Opin. Nephrol. Hypertens. 3, 39–45.

Arunlakshana, O., Schild, H.O., 1959. Some quantitative uses of drug antagonists. Br. J. Pharmacol. 14, 48–58.

Bergman, J., Kamien, J.B., Spealman, R.D., 1990. Antagonism of cocaine self-administration by selective dopamine D1 and D2 antagonists. Behav. Pharmacol. 1, 355–362.

Cai, G., Gurdal, H., Smith, C., Wang, H.-Y., Friedman, E., 1999. Inverse agonist properties of dopamine antagonists at the D1a dopamine receptor: uncoupling of the D1a dopamine receptor from Gs protein. Mol. Pharmacol. 56, 989–996.

Cheng, Y.-C., Prussoff, W.H., 1973. Relationships between the inhibition constant (Ki) and the concentration of inhibitor which causes 50 percent inhibition (I_{50}) of an enzyme reaction. Biochem. Pharmacol. 22, 3099–3108.

Chidiac, P., Hebert, T.E., Valiquette, M., Dennis, M., Bouvier, M., 1994. Inverse agonist activity of β-adrenergic antagonists. Mol. Pharmacol. 45, 490–499.

Chio, C.L., Drong, R.F., Reily, D.T., Gill, G.S., Slinghtom, J.L., Huff, R.M., 1994a. D4 dopamine receptor-mediated signaling events determined in transfected Chinese hamster ovary cells. J. Biol. Chem. 269, 11813–11819.

Chio, C.L., Lajiness, M.E., Huff, R.H., 1994b. Activation of heterologously expressed D3 dopamine receptors: comparison with D2 dopamine receptors. Mol. Pharmacol. 45, 51–60.

Costa, T., Herz, A., 1989. Antagonists with negative intrinsic activity at delta opioid receptors coupled to GTP-binding proteins. Proc. Natl. Acad. Sci. U. S. A. 86, 7321–7325.

DeLean, A., Stadel, J.M., Lefkowitz, R.J., 1980. A ternary complex model explains the agonist-specific binding properties of the adenylate cyclase-coupled β-adrenergic receptor. J. Biol. Chem. 255, 7108–7117.

Djamgoz, M.B.A., Wagner, H.-J., 1992. Localization and function of dopamine in the adult vertebrate retina. Neurochem. Int. 20, 139–191.

Karlsson, P., Smith, L., Farde, L., Harnryd, C., Sedvall, G., Wiesel, F.-A., 1995. Lack of apparent antipsychotic effect of the D1-dopamine receptor antagonist SCH39166 in acutely ill schizophrenic patients. Psychopharmacology 121, 309–316.

Lachowicz, J.E., Sibley, D.R., 1997. Molecular characteristics of mammalian dopamine receptors. Pharmacol. Toxicol. 81, 105–113.

Laitinen, J.T., 1993. Dopamine stimulates K^+ efflux in the chick retina in D1 receptors independently of adenylyl cyclase activation. J. Neurochem. 61, 1461–1469.

Lidow, M.S., Goldman-Rakic, P.S., 1994. A common action of clozapine, haloperidol and remoxipride on D1- and D2-dopamine receptors in the primate cerebral cortex. Proc. Natl. Acad. Sci. 91, 4353–4356.

Lidow, M.S., Elsworth, J.D., Goldman-Rakic, P.S., 1997. Down-regulation of the D1 and D5 dopamine receptors in the primate prefrontal cortex by chronic treatment with antipsychotic drugs. J. Pharmacol. Exp. Ther. 281, 597–603.

Liu, L.-X., Monsma, F.J., Sibley, D.R., Chiodo, L.A., 1996. D2L, D2S and D3 dopamine receptors stably transfected into NG108-15 cells couple to a voltage-dependent potassium current via distinct G proteins. Synapse 24, 156–164.

Luedtke, R.R., Griffin, S.A., Conroy, S.S., Jin, X., Pinto, A., Sesack, S.R., 1999. Immunoblot and immunohistochemical comparison of murine monoclonal antibodies specific for the rat D1a and D1b dopamine receptor subtypes. J. Neuroimmunol. 101, 170–187.

Magar, M.E., 1972. Data Analysis in Biochemistry and Biophysics. Academic Press, New York, NY.

McGonigle, P., Huff, R.M., Molinoff, P.B., 1984. A comprehensive method for the quantitative determination of dopamine receptor subtypes. Ann. N. Y. Acad. Sci. 430, 77–90.

Nestler, E.J., 1994. Hard target: understanding dopaminergic neurotransmission. Cell 79, 923–925.

Salmi, P., 1998. Independent roles of D1 and D2/D3 receptors in rat thermoregulation. Brain Res. 781, 188–193.

Salomon, Y., Londos, C., Rodbell, M., 1974. A highly sensitive adenylate cyclase assay. Anal. Biochem. 58, 541–548.

Samana, P.S., Cotechia, S., Costa, T., Lefkowitz, R.J., 1993. A mutation-induced activated state of the β_2-adrenergic receptor extending the ternary complex model. J. Biol. Chem. 268, 4625–4636.

Scatchard, G., 1949. The attractions of proteins for small molecules and ions. Ann. N. Y. Acad. 51, 660–672.

Schwartz, J.C., Diaz, J., Bordet, R., Griffon, N., Perachon, S., Pilon, C., Ridray, S., Sokoloff, P., 1998. Functional implications of multiple dopamine receptor subtypes: the D1/D3 receptor coexistence. Brain Res. Rev. 26, 236–242.

Self, D.W., Stein, L., 1992. The D1 agonists SKF 82958 and SKF 77434 are self-administered by rats. Brain Res. 582, 349–352.

Sokoloff, P., Giros, B., Martres, M.-P., Bouthenet, M.-L., Schwartz, J.-C., 1990. Molecular cloning and characterization of a novel dopamine receptor (D3) as a target for neuroleptics. Nature 347, 146–149.

Summers, M.D., Smith, G.E., 1987. A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures. Texas Agricultural Experiment Station Bulletin, vol. 1555.

Sunahara, R.K., Guan, H.-C., O'Dowd, B.F., Seeman, P., Laurier, L.G., Ng, G., George, S.R., Torchia, J., Van Tol, H.H.M., Niznik, H.B., 1991. Cloning of the gene for a human D5 receptor with higher affinity for dopamine than D1. Nature 350, 614–618.

Surmeier, D.J., Bargas, J., Hemmings Jr., H.C., Nairn, A.C., Greegard, P., 1995. Modulation of calcium currents by a D1 dopaminergic protein kinase/phosphatase cascade in rat neostriatal neurons. Neuron 14, 385–397.

Tiberi, M., Caron, M.G., 1994. High agonist-independent activity is a distinguishing feature of the dopamine D1B receptor subtype. J. Biol. Chem. 269, 27925–27931.

Tiberi, M., Jarvie, K.R., Silvia, C., Falardeau, P., Ginrich, J.A., Godinot, M., Bertrand, L., Yang-Feng, T.L., Fremeau Jr., R.T., Caron, M.G., 1991. Cloning, molecular characterization and chromosomal assignment of a gene coding a second D1 dopamine receptor subtype: differential expression pattern in rat brain compared with the D_{1A} receptor. Proc. Natl. Acad. Sci. U. S. A. 88, 7491–7495.

Vallar, L., Muca, C., Magni, M., Albert, P., Bunzow, J., Meladolesi, J.,

Civelli, O., 1990. Differential coupling of dopaminergic D2 receptors expressed in different cell types. J. Biol. Chem. 265, 10320–10326.

Ward, A.S., Li, D.H., Luedtke, R.R., Emmett-Ogelsby, M.W., 1996. Variations in the cocaine self-administration of inbred strains of rats using a progressive ratio schedule. Psychopharmacology 127, 204–212.

Williams, G.V., Goldman-Rakic, P.S., 1996. Modulation of memory fields by dopamine D1 receptors in prefrontal cortex. Nature 376, 572–575.

Woolverton, W.L., Virus, R.M., 1989. The effects of a D1 and a D2 dopamine antagonist on behavior maintained by cocaine or food. Pharmacol., Biochem. Behav. 32, 691–699.

Xu, M., Hu, X.T., Cooper, D.C., Moratall, R., Graybiel, A.M., White, F.J., Tonegawa, S., 1994. Elimination of cocaine-induced hyperactivity and dopamine-mediated neurophysiological effects in dopamine D1 receptor mutant mice. Cell 79, 945–955.