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Antagonism of the effects of (+)-PD 128907 on midbrain dopamine neurones in rat brain slices by a selective D_2 receptor antagonist L-741,626

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1 The ability of PD 128907 to activate dopamine receptors in the ventral tegmental area, substantia nigra pars compacta, and striatum was investigated by use of *in vitro* electrophysiological recording and fast cyclic voltammetry. The affinity of a novel D_2 selective antagonist L-741,626 for receptors activated by this agonist was measured to determine if its effects were mediated by D_2 or D_3 receptors.

2 The active (+) enantiomer of PD 128907 bound with high affinity and selectivity to rat D_3 dopamine receptors. The K_i values for (+)-PD 128907 were 620 nM at D_2, 1 nM at D_3 and 720 nM at D_4 receptors.

3 (+)-PD 128907 inhibited cell firing in both the ventral tegmental area and substantia nigra pars compacta with EC_{50} values of 33 nM ($pEC_{50} = 7.48 \pm 0.10$, $n = 10$) and 38 nM ($pEC_{50} = 7.42 \pm 0.15$, $n = 5$), respectively. No effects of (+)-PD 128907 (100 nM) were observed on glutamate or GABA-mediated synaptic potentials elicited by focal bipolar stimulation.

4 L-741,626 antagonized these effects of (+)-PD 128907 in a concentration-dependent and surmountable manner with an affinity, determined from Schild analysis, of 20 nM ($pK_B = 7.71 \pm 0.14$) in the ventral tegmental area and 11 nM ($pK_B = 7.95 \pm 0.18$) in the substantia nigra pars compacta.

5 (+)-PD128907 also inhibited dopamine release in the caudate-putamen with an EC_{50} of 66 nM ($n = 5$). The affinity of L-741,626 for these nerve terminal autoreceptors ($pK_B = 7.71 \pm 0.06$; = 20 nM) was identical to that observed on midbrain dopamine neurones.

6 These data demonstrate that the D_3 receptor ligand (+)-PD 128907 is a potent agonist on rat midbrain dopamine neurones. However, its lack of regional selectivity, and the high affinity of the selective D_2 receptor antagonist L-741,626 for receptors activated by (+)-PD 128907, was more consistent with an action on D_2 autoreceptors rather than upon a D_3 dopamine receptor subtype.

Keywords: D_3 dopamine receptor; schizophrenia; quinpirole; dopamine; ventral tegmental area; substantia nigra; electrophysiology

Introduction

Dopamine receptors are characterized by functional criteria into $D_{1\text{-like}}$ receptors which activate adenylate cyclase and $D_{2\text{-like}}$ receptors which can inhibit adenylate cyclase activity and exert inhibitory influences upon neuronal excitability (Kebabian & Calne, 1979). Activation of $D_{2\text{-like}}$ receptors inhibits cell activity in both the substantia nigra pars compacta (A9) and ventral tegmental area (A10) by hyperpolarizing cells following the activation of G-proteins linked to the activation of potassium channels (Pinnock, 1983; Lacey *et al.*, 1987; 1988; Bowery *et al.*, 1994). The release of dopamine from nerve terminals within the striatum is similarly subject to feedback regulation by $D_{2\text{-like}}$ dopamine autoreceptors (Bull *et al.*, 1990; Stamford *et al.*, 1991).

The recent identification of several structural subtypes of $D_{2\text{-like}}$ dopamine receptors, namely the $D_{2\text{-like}}$, D_3 and D_4 subtypes (Bunzow *et al.*, 1988; Sokoloff *et al.*, 1990; Van Tol *et al.*, 1991) has prompted speculation that these receptors have different functional roles in the central nervous system. This suggestion is supported by the fact that D_3 and D_4 receptor mRNA have a more restricted distribution within brain tissue. In particular D_3 receptors appear to be preferentially expressed within the mesolimbic system (Bouthenet *et al.*, 1991; Levesque *et al.*, 1992) and heterologous expression studies have shown that these receptors can couple to effector mechanisms similar to the D_2 receptor subtype (Chio *et al.*, 1994; Seabrook *et al.*, 1994a,b; Tang *et al.*, 1994; McAllister *et al.*, 1995). Consequently this study was carried out to determine whether D_3 dopamine receptors are functional autoreceptors in rat brain. With this objective in mind the effects of a selective D_3 receptor agonist (+)-PD 128907 (DeWald *et al.*, 1990; DeMattos *et al.*, 1993) on A9 and A10 dopamine neurones was studied, and the ability of a novel D_2 receptor antagonist L-741,626 (Kulagowski *et al.*, 1996) to block these effects were compared.

Methods

[^{3}H]-spiperone binding assay

Cells expressing the rat D_2 (CHO, Dr Sokoloff, INSERM), rat D_3 (rat fibroblasts, Dr Sokoloff, INSERM) and rat D_4 (mouse fibroblasts, Dr Todd, St. Louis, U.S.A.) receptors were lysed by homogenization (polytron, 2 × 5 s) in 10 mM Tris HCl buffer (pH 7.4) containing 5 mM $MgSO_4$ and spun at 50,000 *g* for 15 min. The resulting pellet was resuspended in assay buffer (50 mM Tris.HCl, pH 7.4 containing 5 mM EDTA, 1.5 mM $CaCl_2$, 5 mM $MgCl_2$, 5 mM KCl, 120 mM NaCl, 0.1% ascorbic acid) at a protein concentration of 0.1-0.4 mg ml^{-1} (rat D_2 and D_3 cells) or 2.4 mg ml^{-1} (rat D_4 cells). For the radioligand binding assays, 0.2 nM [^{3}H]-spiperone was used, 50 µl of displacing drugs (0.01 nM to 10 µM) and either 50 µl buffer (total binding) or 10 µM apomorphine (non-specific

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binding) in a final assay volume of 500 μl. The reaction was initiated by the addition of 75 μl of membranes and allowed to proceed for 2 h at room temperature before being terminated by rapid filtration over GF/B filters (presoaked in 0.3% PEI) with 2 × 5 ml ice cold 50 mM Tris.HCl, pH 7.4. Binding parameters were determined by non-linear, least squares regression analysis using RS1 (BBN Research Systems, Cambridge, Mass., U.S.A.) and an in-house computerized iterative procedure developed by Dr A. Richardson.

Electrophysiological preparations and recording techniques

Extracellular and intracellular recordings were made from presumed dopamine-containing neurones in the ventral tegmental area and substantia nigra pars compacta within slices (300–350 μm thick) of rat brain (male, Sprague Dawley). The methods used were as previously described (Bowery *et al.*, 1994). In brief, coronal slices of brain (plate 25, Paxinos & Watson, 1994) were submerged in a tissue chamber (volume 0.4 ml) by a continuously superfused (at 1–2 ml min^{-1}) artificial cerebrospinal fluid (ACSF) kept at 36°C and gassed with 95% O_2 and 5% CO_2. The ACSF contained (in mM): NaCl 126, KCl 2.5, NaH_2PO_4 1.2, $MgCl_2$ 1.3, $CaCl_2$ 2.4, $NaHCO_3$ 26 and glucose 10. Extracellular electrophysiological recordings were made with glass microelectrodes filled with 3 M NaCl (5–10 MΩ). Intracellular recordings were made with glass microelectrodes that had been filled with 3 M KCl (10–40 MΩ).

Dopamine release from within slices (300–350 μm thick) of rat striatum (caudate-putamen) was measured by *in vitro* fast cyclic voltammetry. The methods used were as previously described (Bull *et al.*, 1990). The oxidation peak for dopamine was set at +700 mV and release was evoked by a single 0.1 ms pulse of 5 volts every 2 min via a tungsten bipolar stimulating electrode. Cumulative concentration-effect curves were obtained with quinpirole, and (+)-PD 128907 (0.01 to 10 μM) in the absence and presence of L-741,626 (30, 100 and 300 nM). Cocaine (1 μM) was present in the ACSF throughout the experiments to reduce catecholamine uptake processes. Without the addition of cocaine to maximize the electrically-induced dopamine release, the signal to noise of the voltammetric recordings was too weak in our hands to construct reliably concentration-effect curves to the agonist.

Analytical techniques

Drug effects were expressed as either the % inhibition of cell firing or % inhibition of dopamine release. Agonist-induced inhibition of cell firing was determined by use of cumulative concentration-effect curves, 7 min was allowed for the firing rate to stabilize in each concentration of the agonist. Agonist potency was quantified as the negative logarithm of the half-maximal inhibitory concentration (pIC_{50}), and pooled data are the geometric mean ± s.e. mean. Concentration-effect data were fitted to a logistic equation, $A/(1+(B/X)^C)$ where A = maximum, B = EC_{50}, C = Hill slope, X = agonist concentration, by least squares analysis of variance (Grafit; Erithacus Software). Antagonist affinities were determined by Schild analysis (Arunlakshana & Schild, 1959), using the shift in the concentration of the agonist required to half-maximally inhibit cell firing in the absence and presence of increasing concentrations of the antagonist. In experiments where the Schild plot had a slope that was not significantly differ from unity, the antagonist affinity (pK_B value) was calculated by fitting a line with a slope of one to the data (Kenakin, 1990).

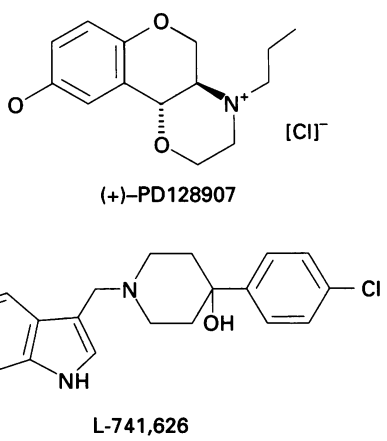

Figure 1 Chemical structures of (+)-PD 128907 and L-741,626.

Drugs

Drugs were applied in the superfusion medium in known concentrations, reaching the recording chamber after a delay of 20–30 s. The following drugs were used: quinpirole hydrochloride (Research Biochemicals Inc), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; Tocris Neuroamin), (+)-PD 128907 (R-(+)-4,4a,10b-tetrahydro-4-propyl-2*H*,5*H*-[1]benzopyrano[4,3-*b*]-1,4-oxazin-9-ol) and L-741,626 (3-[4-(4-chloro)phenyl-4-hydroxypiperidino)methyl]indole) (Kulagowski *et al.*, 1996). (+)- and (−)-PD 128907, and L-741,626 were synthesised at Merck Sharp & Dohme Research Laboratories. Their chemical structures are shown in Figure 1. Where necessary drugs were dissolved in dimethylsulphoxide (DMSO), and diluted to a final bath concentration of <0.1% DMSO which alone had no effect on cell firing rates.

Results

Binding studies

The receptor binding kinetics and parameters of [^{3}H]-spiperone binding to D_2, D_3, and D_4 receptors were fully characterized and some of these data have been published previously (Freedman *et al.*, 1994; McAllister *et al.*, 1995). In the rat clones [^{3}H]-spiperone bound specifically and saturably, with dissociation constants of 0.05 ± 0.01 nM (n = 4), 0.37 ± 0.03 (n = 3),

Table 1 Affinities of the ligands used in the present study for cloned rat $D_{2(short)}$, D_3 and D_4 dopamine receptors

Ligand	Receptor subtype D_2	D_3	D_4
Quinpirole			
K_i (nM)	>1990 (43%)	16 (12, 22)	110 (74, 160)
n_H	–	0.82 ± 0.10	0.71 ± 0.02
(+)-PD 128907			
K_i (nM)	620 (420, 1400)	0.99 (0.8, 1.2)	720 (360, 1500)
n_H	0.91 ± 0.07	0.84 ± 0.05	1.20 ± 0.07
(−)-PD 128907			
K_i (nM)	>2200 (6%)	5800 (5300, 6300)	>5300 (28%)
n_H	–	1.10 ± 0.001	–
L-741,626			
K_i (nM)	11.5 (8.7, 15)	120 (90, 160)	750 (746, 748)
n_H	0.98 ± 0.07	0.92 ± 0.15	1.20 ± 0.09

Results are expressed as the K_i value (nM) and numbers in parentheses indicate the low and high errors of the geometric mean. The IC_{50} values were determined from 3 to 4 separate experiments where each curve consisted of 5 to 6 points, each in triplicate. The IC_{50} values were corrected with the Cheng Prusoff equation (Cheng & Prusoff, 1973). The Hill coefficients (n_H) are expressed as the mean ± s.e.mean.

and 0.25 ± 0.04 nM ($n=3$) for D_2, D_3 and D_4 receptors, respectively. Receptor densities (Bmax values) for D_2, D_3 and D_4 rat clones were approximately 1800, 1600 and 250 fmol mg^{-1} protein, respectively. The binding profile of (+)-PD128907, (−)-PD128907, quinpirole, and L-741,626 at rat dopamine D_2, D_3 and D_4 receptors is shown in Table 1. (+)-PD128907 bound with high affinity ($Ki = 1$ nM) and 600 fold selectivity for the rat dopamine D_3 receptor whilst its entantiomer (−)-PD128907 either did not bind or had lower affinity at all the rat dopamine receptor subtypes examined. Quinpirole bound with relatively low affinity to the D_2 subtype (*ca.* 2 μM) compared to the D_3 subtype. (+)-PD 128907 had approximately 16 fold higher affinity for D_3 receptors than quinpirole. The selective D_2 receptor antagonist, L-741,626 exhibited >10 fold selectivity for rat D_2 over D_3 receptors (Ki at $D_2 = 11$ nM; $D_3 = 120$ nM). All four compounds showed only relatively weak affinity for rat D_4 dopamine receptors (Table 1).

Table 2 Potency of agonists in the ventral tegmental area (VTA) and substantia nigra pars compacta (SNC) compared with their potency at inhibiting dopamine release in the caudate-putamen (CPu)

	VTA	*SNC*	*CPu*
Quinpirole			
IC_{50}	58 nM	22 nM	66 nM
pIC_{50}	7.24±0.06	7.65±0.06	7.18±0.16
n	(32)	(35)	(5)
(+)-PD 128907			
IC_{50}	33 nM	38 nM	112 nM
pIC_{50}	7.48±0.10	7.42±0.15	6.95±0.07
n	(10)	(5)	(6)
(−)-PD 128907			
IC_{50}	>300 nM	>300 nM	NT
pIC_{50}	<6.5	<6.5	NT
n	(3)	(3)	

Agonist responses were qualified as the negative logarithm of the half-maximal inhibitory concentration (pIC_{50}). Data are geometric mean±s.e.mean. NT=not tested. Numbers in parentheses are number of cells/preparations examined.

Functional effects of (+)-PD 128907

In functional assays (+)-PD 128907 was a potent agonist on both A9 and A10 neurones. Within the ventral tegmental area (VTA) (+)-PD128907 caused a potent and reversible inhibition of cell firing with a pEC_{50} of 7.48±0.10 (equivalent to 33 nM, $n=10$) compared to quinpirole whose EC_{50} was 58 nM (Table 2; Bowery *et al.*, 1994). This was a consequence of an increase in membrane conductance and hyperpolarization (Figure 2a).

A comparison between the ability of (+)-PD 128907 to inhibit action potential firing in the substantia nigra pars

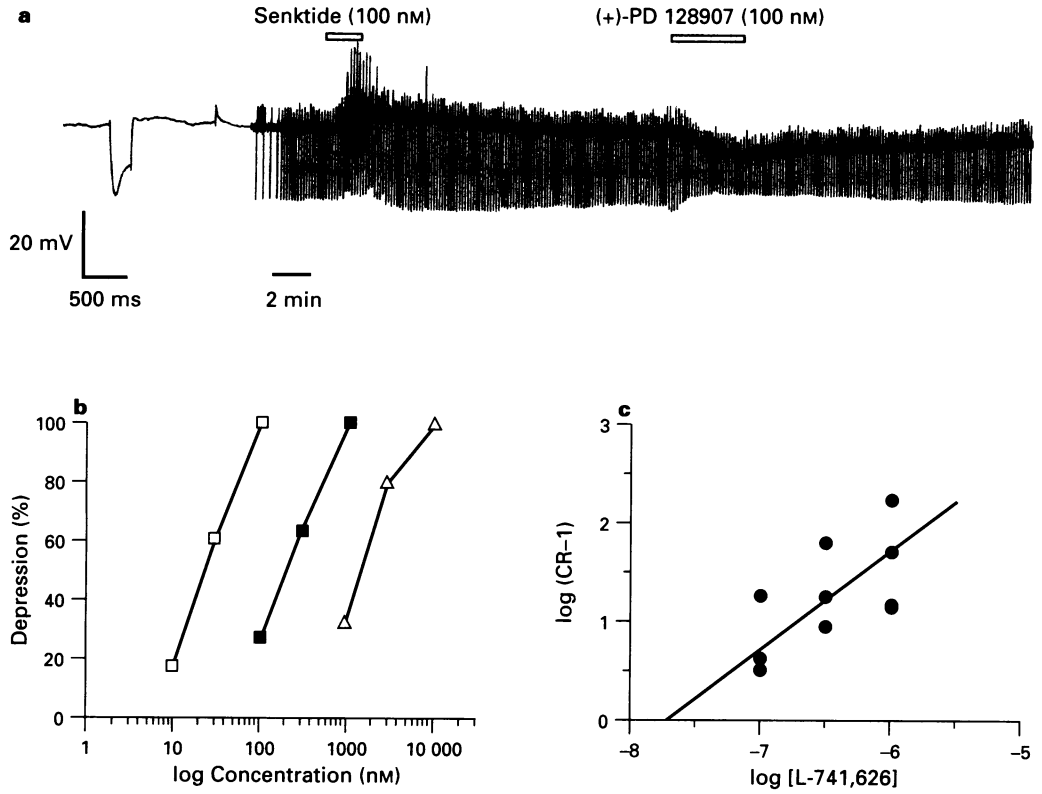

Figure 2 Inhibition of cell firing in the ventral tegmental area by (+)-PD 128907 and its antagonism by L-741,626. (a) Intracellular recording of the membrane potential in an individual neurone contrasting the excitation caused by the NK_3 tachykinin receptor agonist, senktide (100 nM) and hyperpolarization caused by (+)-PD 128907 (100 nM). Hyperpolarizing current steps (−0.2 nA for 300 ms), reflected by the downward deflections, were applied every 10 s to monitor the cell's input resistance. Application (+)-PD 128907 (100 nM), for the duration of the horizontal bar, produced a reversible hyperpolarization of the cell membrane potential from −61 mV to −72 mV, and this was accompanied by an increase in membrane conductance. (b) The spontaneous firing of action potentials by these neurones, measured with extracellular electrodes, was also inhibited in a concentration-dependent manner by (+)-PD 128907 (□; see Table 2). L-741,626 blocked the effects of low concentrations of (+)-PD 128907, an effect that was overcome by increasing the agonist concentration. (■), L-741,626 100 nM, and (△), L-741,626 300 nM, represent concentration-effect curves for (+)-PD 128907 in the presence of the antagonist. (c) The shift in the half maximally effective concentration of (+)-PD 128907 for increasing concentrations of L-741,626 was used to generate a Schild plot. The pA_2 value for L-741,626 was 7.95 with a Hill slope of 1.0 consistent with competitive antagonism. Thus the affinity (pK_B) of L-741,626 for the receptors activated by (+)-PD128907 is equal to its pA_2 value. The standard error of the pK_B was determined by constraining the Schild plot to unity (7.95±0.18).

compacta (SNC) by activating cell body autoreceptors, and to inhibit dopamine release within the striatum by activating terminal autoreceptors was also made. In the SNC, like the VTA, (+)-PD 128907 was a potent agonist with a pEC_{50} of 7.42±0.15 (38 nM). Like quinpirole, the potency of this agonist was slightly weaker (3 fold) at inhibiting dopamine release within the caudate-putamen (CPu), a brain region to which these neurones project (Table 2). The effects of (+)-PD 128907 were stereoselective in that (−)-PD 128907 was inactive at a concentration of 300 nM in all the tissues examined (Table 1).

To investigate whether D_3 receptors are involved in the modulation of synaptic transmission onto midbrain dopamine neurones, like the ability of $GABA_B$ receptors to inhibit γ-aminobutyric acid (GABA) and glutamate excitatory postsynaptic potentials (e.p.s.ps) in the striatum (Seabrook *et al.*, 1990), the ability of PD 128097 to inhibit synaptic potentials in VTA neurones was studied by intracellular recording techniques (Figure 3). (+)-PD 128907 (100 nM) hyperpolarized VTA neurones via an increase in membrane conductance, with a maximum hyperpolarization (7±1 mV, 5 cells) that was comparable to that of quinpirole (Figure 2a; Bowery *et al.*, 1994). Focal bipolar stimulation within the slice elicited e.p.s.ps with amplitudes of 7.0±1.3 mV (held at −68±3 mV, $n=11$). These e.p.s.ps were reduced in amplitude to 65±11% ($n=9$) of control by the glutamate receptor antagonist CNQX (30 μM), and to 16±2% ($n=5$) by co-application of CNQX and the $GABA_A$ receptor antagonist (−)-bicuculline methodide (both 30 μM). (+)-PD 128907 (100 nM) did not affect the amplitude of the mixed glutamate / GABA synaptic potentials (102% of control, $n=4$), or that of pharmacologically isolated GABA-mediated synaptic potentials which were recorded in the presence of CNQX (96% of control, $n=4$), or glutamate e.p.s.ps recorded in the presence of bicuculline (Figure 3d).

Antagonism by L-741,626

In both the SNC and VTA the inhibition of cell firing caused by quinpirole was blocked in a surmountable manner by L-741,626. In the SNC the pA_2 value for L-741,626 versus quinpirole was 7.95 with a slope not significantly different from unity (0.99±0.45). Constraining the slope to one gave a pK_B value of 8.00±0.18, equivalent to an affinity of 10 nM. Similarly in the VTA the pA_2 value for L-741,626 versus quinpirole was 7.64 (nH = 0.74±0.20), yielding a pK_B value of 7.30±0.07 (50 nM).

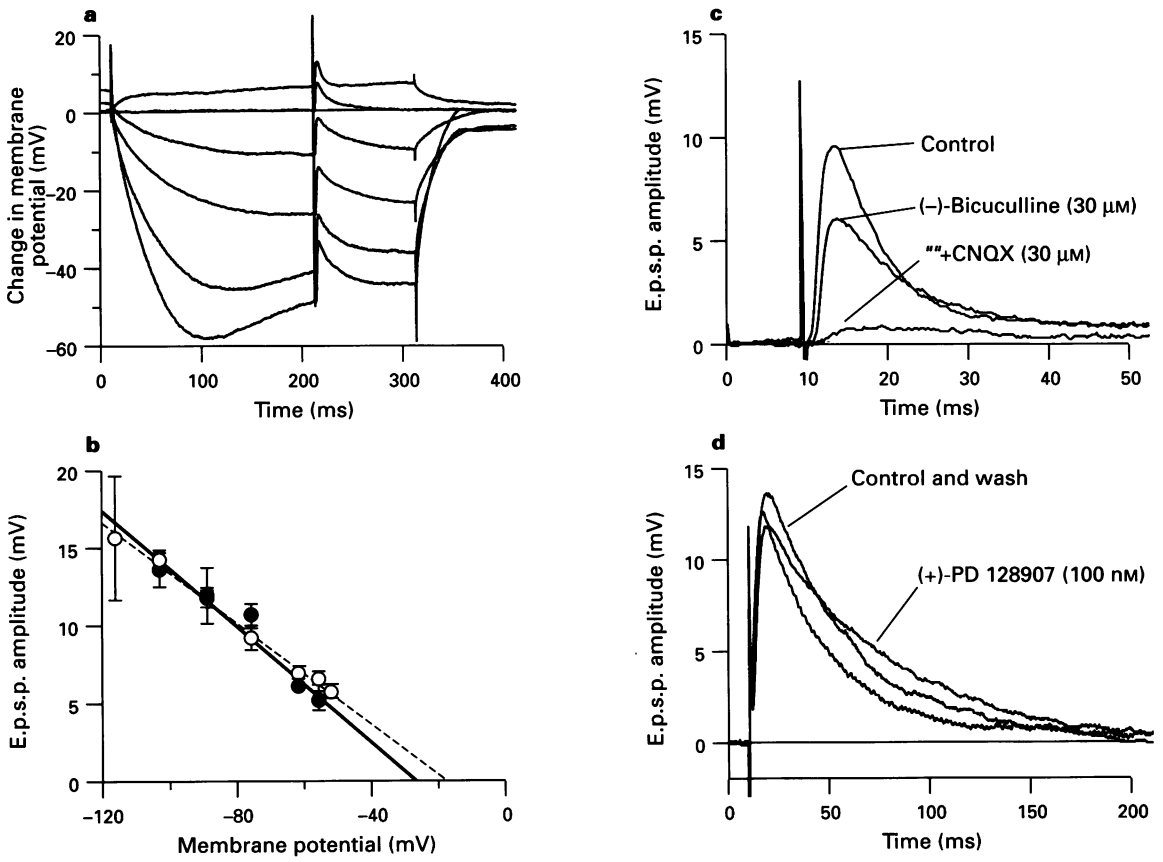

Figure 3 Effect of PD 128907 on synaptic potentials in ventral tegmental area. (a) Current-voltage relationship from an individual neurone (held at −60 mV by constant injection of −0.03 nA current to prevent cell firing) measured using intracellular recording electrodes. Current steps were injected via the recording electrode, from −0.3, −0.2, −0.1, −0.05 to +0.05 nA for 300 ms, and the resultant change in membrane potential was used to measure the cell input resistance. This was 130 MΩ over the ohmic region of the I/V curve. Note the time-dependent inward rectification, which is characteristic of dopamine neurones (Lacey *et al.*, 1988), caused by transients to negative membrane potentials. E.p.s.ps were elicited 200 ms into the current step so that their amplitude at different membrane potentials could be used to estimate their reversal potential. (b) In this cell the estimated reversal potential of the control e.p.s.p was −17 mV (○) and in the presence of CNQX (30 μM), used to block glutamate-mediated synaptic potentials, was shifted negatively to −26 mV and approached that predicted for the Cl^- equilibrium potential based on intracellular electrodes filled with 3 M KCl. (c) Superimposed synaptic potentials from another cell during control conditions, after application of bicuculline (30 μM) to block $GABA_A$ mediated potentials, and after co-application of bicuculline plus CNQX (30 μM). Despite the pronounced hyperpolarizations caused by (+)-PD 128907 (e.g. Figure 1) no significant effects were observed on the amplitude of fast synaptic potentials in these cells. (d) Superimposed e.p.s.ps from a different cell which had been pretreated with bicuculline to isolate the glutamate-mediated e.p.s.ps (+)-PD 128907 (100 nM) had no significant effect on the peak amplitude of these e.p.s.ps, although the slight prolongation in the rate of decay of the e.p.s.p. in PD 128907 was accounted for by the postsynaptic hyperpolarization (see (a)).

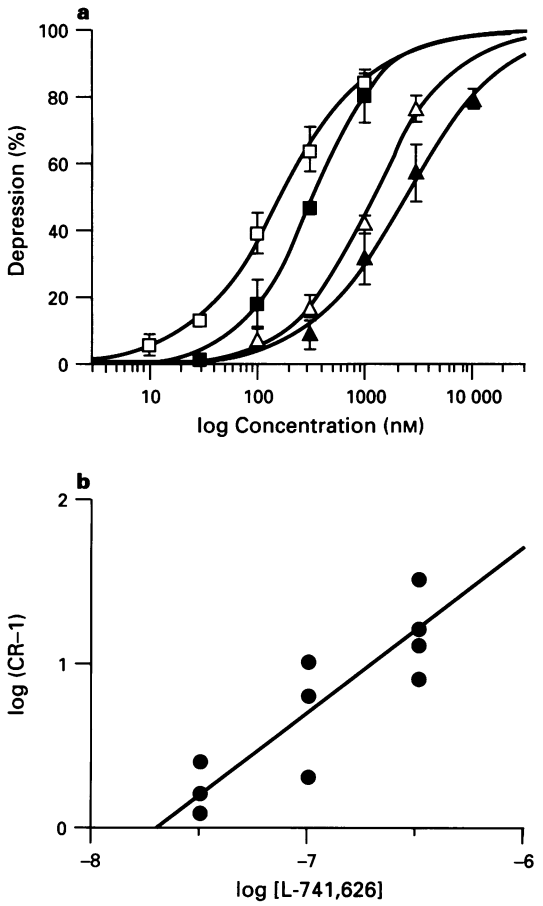

Figure 4 Inhibition of dopamine release in caudate-putamen by (+)-PD 128907 and its antagonism by L-741,626. (a) Dopamine release was inhibited in a concentration-dependent manner by (+)-PD 128907. L-741,626 blocked the effects of (+)-PD 128907, an effect that was overcome by increasing the agonist concentration. □, Control; ■, 30 nM L-741,626; △, 100 nM L-741,626; ▲, 300 nM L-741,626. Data are the mean ± s.e. mean > 3 preparations. Solid curves represent logistic equation fitted to the data by least squares analysis of variance. (b) As in Figure 2 the shift in the half-maximally effective concentration of (+)-PD 128907 with increasing concentrations of L-741,626 was used to generate a Schild plot. The pA_2 value for L-741,626 was 7.77 with a Hill Slope of 0.95 ± 0.16 consistent with competitive antagonism. The affinity of L-741,626 (pK_B) was determined by constraining the slope of the Schild plot to unity (solid line; 7.71 ± 0.06).

The effects of (+)-PD 128907 in both the SNC and VTA, like those of quinpirole, were antagonized by L-741,626 in a competitive and surmountable manner. In the SNC the pA_2 value for L-741,626 versus (+)-PD 128907 was 7.47 with a slope not significantly different from unity (1.50 ± 0.43). Constraining the slope to one gave a pK_B value of 7.95 ± 0.18, equivalent to an affinity of 11 nM. The pA_2 value for L-741,626 versus (+)-PD 128907 in the VTA was 8.11 (nH = 0.74 ± 0.33; Figure 2c), with a pK_B value of 7.71 ± 0.14 (= 20 nM).

The ability of (+)-PD 128907 to inhibit electrically stimulated dopamine release in the caudate putamen was also antagonized by L-741,626 (Figure 4). The Schild plot for L-741,626 gave a pA_2 value of 7.77 with a Hill slope of 0.95 ± 0.16. Constraining the slope to one yielded a pK_B of 7.71 ± 0.06 equivalent to an affinity of 20 nM (Table 2).

Discussion

By use of *in vitro* electrophysiological recording, this study examined the ability of a D_3-selective ligand (+)-PD 128907 to inhibit spontaneous action potential firing of midbrain dopamine neurones in rat brain slices. We have previously demonstrated that the inhibition of cell firing caused by quinpirole in the SNC and VTA is mediated by a population of receptors that have low affinity for S(−)-sulpiride and clozapine, and thus are unlikely to be D_4 dopamine receptors (Bowery *et al.*, 1994). To investigate further the pharmacology of dopamine receptors on midbrain dopamine neurones, and in particular to determine whether D_3 receptors are functional dopamine autoreceptors, the effects of a novel and selective D_2 receptor antagonist on responses to PD 128907 were examined.

Table 3 Affinity of L-741,626 for receptors activated by quinpirole and (+)-PD 128907 in the ventral tegmental area (VTA) substantia nigra pars compacta (SNC) and caudate-putamen (CPu)

	VTA	*SNC*	*CPu*
L-741,626 v. quinpirole	7.30 ± 0.07 = 50 nM (36, 69)	8.00 ± 0.18 = 10 nM (4, 23)	NT
L-741,626 v. PD 128907	7.71 ± 0.14 = 20 nM (10, 37)	7.95 ± 0.18 = 11 nM (5, 26)	7.71 ± 0.06 = 20 nM (15, 26)

The affinity of L-741,626 was determined by Schild analysis. Data are the negative logarithm of the antagonist dissociation constant (pk_B) determined by constraining the slope of the Schild plot to one. Numbers in parentheses are the 95% confidence limits of the geometric mean (in nM). NT = not tested.

Several histological techniques have been used to localize D_3 receptors in rat brain, including *in situ* hybridization and radioligand binding studies with the selective ligand 7-hydroxy-N,N,di-n,propyl-2-aminotetralin (7-OH-DPAT; Bouthenet *et al.*, 1991 Levesque *et al.*, 1992). From these studies it is apparent that both D_3 mRNA and binding sites for [^{3}H]-7-OH-DPAT are located preferentially within the mesolimbic system, most notably within the shell of the nucleus accumbens, the olfactory tubercle and Islands of Calleja. Interestingly some mRNA for D_3 receptors is also found within the ventral tegmental area and substantia nigra of rat brain (Sokoloff *et al.*, 1990). Although D_3 receptors are more restricted in their distribution compared to their D_2 counterparts, several studies have shown that these receptors can couple to G-proteins that link to similar effector mechanisms that include the regulation of calcium currents and inhibition of adenylate cyclase (Seabrook *et al.*, 1994a; Chio *et al.*, 1994; McAllister *et al.*, 1995). $D_{2\text{-like}}$ dopamine receptors in midbrain dopamine neurones couple to G-proteins that regulate cell firing by the activation of potassium currents and concomittant membrane hyperpolarization. Thus if D_3 receptor mRNA is translated into functional receptor protein these receptors may serve as autoreceptors that provide feedback regulation on dopamine cell activity.

The benzopyranoxazine PD 128907 was originally identified as a relatively weak D_2 dopamine receptor ligand (De Wald *et al.*, 1990), but has since been shown to have high affinity and selectivity for cloned D_3 dopamine receptors (DeMattos *et al.*, 1993). Furthermore, in functional assays this ligand is an agonist at cloned human D_3 receptors (McAllister *et al.*, 1995) and also modulates midbrain dopamine cell function (DeWald *et al.*, 1990). Estimates of the binding selectivity of agonists for D_3 over D_2 receptors are influenced by whether one compares the high or low affinity states of the receptors (e.g. Burris *et al.*, 1995). In the present study [^{3}H]-spiperone preferentially labelled the low affinity sites at which approximately 600 fold selectivity for D_3 receptors was seen. D_3 receptors exhibit limited guanosine 5'-triphosphate (GTP) sensitive agonist binding compared to the D_2 subtype (e.g. Sokoloff *et al.*, 1990; Freedman *et al.*, 1994; Pugsley *et al.*, 1995) therefore these values can be considered to be an upper limit of the binding selectivity for (+)-PD 128907 on D_3 versus D_2 receptors.

However, when displacement of high affinity receptor binding sites is examined (+)-PD 128907 remains at least 14 fold selective for the D_3 receptor subtype (Pugsley *et al.*, 1995). *In vivo*, low doses of (+)-PD 128907 reduce spontaneous locomotor activity in rats and impair prepulse inhibition of the acoustic startle reflex (Bristow *et al.*, 1996). Potent effects of another D_3 receptor agonist 7-OH-DPAT are also observed on rat midbrain dopamine neurones (Bowery *et al.*, 1994), and in a recent study it was concluded that this may be due to the activation of D_3 autoreceptors (Leujeune & Milan, 1995). Similarly the rank order of potency of dopamine agonists to inhibit cell firing in the VTA *in vivo* has been correlated with their D_3 receptor affinity (Kriess *et al.*, 1995). Using 7-OH DPAT, Aretha and colleagues have also implicated D_3 receptors as dopamine synthesis regulating autoreceptors in the olfactory tubercle (Aretha *et al.*, 1995). But it should be noted that such comparisons are based primarily upon *in vitro* binding affinities and thus may be compromised by the intrinsic activity (efficacy), receptor reserve, and/or pharmacokinetics of a given drug (Seabrook *et al.*, 1995).

In the present study (+)-PD 128907 was a potent agonist on neurones within both the SNC and VTA. Despite the higher affinity of (+)-PD 128907 for D_3 receptors the potency of this agonist was identical to that of quinpirole in both brain areas. Like quinpirole, the potency of (+)-PD 128907 was slightly weaker (3 fold) at inhibiting dopamine release within the caudate-putamen than in the substantia nigra, a brain region to which these neurones project (Table 2). However, it should be noted that inhibition of dopamine uptake with cocaine in the voltammetry studies may lead to the extracellular accumulation of endogenous dopamine which could preclude the determination of accurate potency values for these agonists (Lacey *et al.*, 1987). Receptor classification using agonists is further complicated by the influence that receptor reserve and variations in intrinsic activity have upon agonist potency. Thus the similar potency of quinpirole and (+)-PD 128907 may either be because (+)-PD 128907 has a lower efficacy on D_3 receptors compared to quinpirole, and / or that both agonists are acting upon tissues with a high D_2 receptor reserve. Consequently to determine whether the effects of (+)-PD 128907 were mediated by a selective action upon D_3 or D_2 receptors we examined the ability of a novel D_2 receptor antagonist L-741,626 to block its effects.

The indole L-741,626 is a novel high affinity D_2 dopamine receptor ligand that has >10 fold selectivity over D_3 and D_4 dopamine receptors in binding studies (Table 1). In both A9 and A10 brain regions the effects of quinpirole and (+)-PD 128907 were antagonized by L-741,626 demonstrating that this ligand is indeed an antagonist. The affinity of L-741,626 for the receptors activated by both agonists was identical to its affinity for cloned rat D_2 receptors (Tables 1 and 3). Therefore these data probably reflect an effect of agonists on tissues with high D_2 receptor reserves. Likewise the 3 fold greater potency of (+)-PD 128907 on dopamine nerve terminal autoreceptors in the CPu, compared to the SNC, was likely to be a consequence of different receptor reserves as both of these effects were blocked by L-741,626 with equal affinity. The contention that midbrain dopamine neurones have a high dopamine receptor reserve is supported by *in vivo* studies with the irreversible inactivator N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) (Cox & Waszczack, 1990). Similarly autoreceptors regulating dopamine release in the striatum are known to have high receptor reserves (Meller *et al.*, 1986; Yokoo *et al.*, 1988). The ability of L-741,626 to block the effects of (+)-PD 128907 suggests that if D_3 receptors are present on these dopamine neurones it is unlikely that they contribute to the inhibition of action potential firing caused by these exogenously applied agonists. Furthermore, it is unlikely that these data result from effects on a mixed population of D_2 and D_3 receptors because (1) the Hill slopes from the Schild analysis were not significantly different from unity, and (2) the preferential affinity of the agonist used in this study for D_3 receptors would have resulted in residual effects of (+)-PD 128907 that were insensitive to L-741,626 and these were not observed.

Additional evidence that the effects of (+)-PD 128907 were mediated by an action on D_2 and not D_3 receptors is provided by the effects of this agonist on dopamine release from nerve terminals within the caudate-putamen. Histological studies indicate that D_3 receptors are either absent or present in very low density within the CPu compared to D_2 receptors which are similarly expressed in both dorsal and ventral striatal areas (Bouthenet *et al.*, 1991; Levesque *et al.*, 1992). Surprisingly, despite this low density of D_3 receptors in the CPu, (+)-PD 128907 was still an efficacious and potent agonist at inhibiting dopamine release in this brain region. Furthermore L-741,626 antagonised the effects of both (+)-PD 128907 with a identical affinity to that observed with quinpirole, and also with its affinity for receptors in both A9 and A10 neurones.

Interestingly, Kruk and colleagues (Patel *et al.*, 1995) have shown that electrically stimulated dopamine release in the rat nucleus accumbens, a region innervated by A10 dopamine neurones, is inhibited in a biphasic manner by 7-OH DPAT with EC_{50}s of 15 pM and 8 nM, respectively. Whether this biphasic effect can be accounted for by an action on two distinct subtypes of $D_{2\text{-like}}$ dopamine receptors remains to be determined by use of selective D_3 receptor antagonists, as each component was differentially affected by haloperidol which has similar affinity for D_2 and D_3 receptors (Freedman *et al.*, 1994).

The present study highlights the difficulty of interpreting pharmacological studies that use agonists whose selectivities have been defined only in binding assays and not functional assays. (+)-PD 128907 has preferential affinity for D_3 over D_2 receptors yet the potent effects of this agonist on midbrain dopamine neurones were antagonized by L-741,626, and thus it was likely that these effects were mediated by D_2 receptors. The implication that D_3 receptors are not functional autoreceptors in these rat brain regions suggests that selective D_3 receptor antagonists may not necessarily accentuate behaviours that are associated with an increased dopamine turnover in the mesolimbic system, although this remains to be proven. Moreover, if functional D_3 receptors are located on neurones within the ventral striatum, antagonists at these receptors may ameliorate some of the behavioural effects of a hyperactive mesolimbic system, such as that thought to underlie the psychosis associated with schizophrenia (Goldstein & Deutch, 1992; Reynolds, 1992).

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