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Comparative Biochemical Pharmacology of Central Nervous System Dopamine D_1 and D_2 Receptors

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Abstract

The biochemical properties of central nervous system (CNS) dopamine (DA) D_1 and D_2 receptors were examined using the specific antagonists [^{3}H]SCH23390 and [^{3}H]raclopride, respectively. There is a different participation of sulfhydryl (-SH) and disulfide (-SS-) groups in the binding site and/or coupling to second messenger systems of D_1 and D_2 receptors. The ionic studies with [^{3}H]SCH23390 showed slight agonist and antagonist affinity shifts for the D_1 receptor. On the other hand, the D_2 receptor is very sensitive to cations; even if lithium and sodium influence specific [^{3}H]raclopride binding in a similar manner, there appear to be quantitative differences between these two ions that cannot be explained by surface charge mechanisms. The distribution of D_1 and D_2 receptors was heterogenous in both species, with the greatest densities in the neostriatum, where the highest concentrations of DA and metabolites were measured. Regions with low endogenous DA content (cerebral cortex and hippocampus) had lower densities of DA receptors. Furthermore, these binding sites were differentially localized within the various regions, and there were substantially more D_1 than D_2 receptors. The functional significance and heterogeneities in the distribution of D_1 and D_2 receptors can be related to dopaminergic innervation and turnover.

Introduction

Dopamine-containing neurons have been identified within the mammalian CNS. The two major dopaminergic projections, the mesostriatal and mesocortical pathways, originate in the *Substantia nigra* and ventral tegmental area of the mesencephalon and project primarily to the neostriatum and cerebral cortex (i.e., the piriform, entorhinal, cingulate, and medial prefrontal cortical areas), respectively. In the CNS, the neurotransmitter DA regulates a broad spectrum of neurophysiological and neurochemical events, acting through at least two distinct receptors, namely, D_1 and D_2 (Kebabian and Calne, 1979; Seeman, 1980; Creese et al., 1983), that not only present typical pharmacological profiles and particular patterns of distribution within the CNS but in addition are differentially coupled to their second messenger system: adenylate cyclase.

In the past two years, molecular cloning and expression has been possible for both the D_1 (Monsma et al., 1990; Sunahara et al., 1990; Dearry et al., 1990; Zhou et al., 1990) and D_2 (Grandy et al., 1989; Stormann et al., 1989; Chio et al., 1990; Gandelman et al., 1991) receptor subtypes. A third type of DA receptor has been cloned, the D_3, and has been proposed to be the site of action of neuroleptic drugs, although its coupling to a second messenger system has not been fully established (Sokoloff et al., 1990). In addition, more recently, two other DA receptors have been proposed: the D_4 receptor, which has a high homology to human D_2 and D_3 receptor genes (Van Tol et al., 1991), and the D_5 receptor, which shows a strong homology to the D_1 receptor and also stimulates adenylate cyclase but has a higher affinity for DA (Suhanara et al., 1991). Thus, the introduction of molecular biology techniques in pharmacology has enabled numerous groups to determine the base sequences of DNA encoding for the classical D_1 and D_2 receptors as well as to discover new DA sites or receptor subtypes that await further investigation.

On the basis of the homology between the genes cloned to date as well as on their coupling to second messenger systems, it would appear that D_5 receptors can be considered a subgroup of the classical D_1 receptor; D_5 stimulates adenylate cyclase activity and has a pharmacological profile similar to the cloned D_1 receptor but displays a tenfold higher affinity for DA (Sunahara et al., 1991). On the other hand, the D_2, D_3, and D_4 cDNAs may represent subgroups of the gene that codes the classical D_2 receptor site; DA receptors within these groups share similar pharmacological profiles, although small differences are apparent in agonist or antagonist binding (Sokoloff et al., 1990; Van Tol et al., 1991).

Dopamine D_1 receptors are positively coupled by the guanine nucleotide binding protein G_s to adenylate cyclase, and their stimulation increases cAMP levels. On the other hand, dopaminergic D_2 agonists inhibit adenylate cyclase (De Camilli, Macconi, and Spada, 1979; McDonald et al., 1984; Onali, Olianas, and Gessa, 1985) and the formation of cAMP via an inhibitory guanine nucleotide binding protein or G_i protein. It has been also postulated that D_2 receptors exist in two interconvertible affinity states (De Lean, Kilpatrick, and Caron, 1982; Sibley, De Lean, and Creese, 1982; Wreggett and Seeman, 1984; Simmonds et al., 1986) and that receptor sites, when coupled to G-proteins, exhibit high affinity for agonists, whereas those dissociated from the regulatory units remain in the low-affinity state (De Lean, Kilpatrick, and Caron, 1982; Sibley, De Lean, and Creese, 1982; Wreggett and Seeman, 1984). Guanine nucleotides modulate the equilibrium between these two states of the D_2 receptor: D_2^{HIGH} and D_2^{LOW} (MacKenzie and Zigmond, 1984; Grigoriadis and Seeman, 1985; Reader et al., 1990).

In the mid 1970s, it was demonstrated that the antipsychotic action of a series of neuroleptic drugs was highly correlated with their affinity for the striatal DA D_2 receptor, suggesting that the antipsychotic potency of these drugs was mediated through blockade of this site (Seeman et al., 1976; Seeman, 1980; Creese et al., 1983). These and related findings led to the *Dopamine Theory of Schizophrenia*, which in its simplest form, states that this disease may be associated with a relative excess of central dopaminergic neuronal activity and possibly to an increase in central DA D_2 receptor number. The interactions between DA D_1 and D_2 receptors have been described under a number of different behavioral and biochemical paradigms, so that a possible role for D_1 receptor antagonists has been proposed in the treatment of schizophrenia. Several D_1 antagonists have been found to have neuroleptic actions on a series of standard laboratory screening tests. Furthermore, chronic treatment with the atypical antipsychotic agent Clozapine^R has been found to increase D_1 but not D_2 receptor number, suggesting that this drug may interact with the D_1 site (O'Dell et al., 1990). In the context of the possible role of central DA systems in the etiology and treatment of schizophrenia, it is important to understand some of the biochemical and pharmacological properties of DA D_1 and D_2 receptor sites in the CNS. Recent advances in the isolation and characterization of the molecular structure of the D_1 and D_2 receptors and their mRNAs has resulted in the identification of various D_1 and D_2 receptor subtypes (Momsma et al., 1990; Gandelman et al., 1991; Sokoloff et al., 1990). For simplicity, we shall focus this review on D_1 and D_2 receptor sites as defined by their pharmacological profile for a series of dopaminergic agonists and antagonists.

Materials and Methods

Materials

The radiolabeled compounds [^{3}H]SCH23390 (specific activity 60.5 Ci/mmol) and [^{3}H]raclopride (86.9 Ci/mmol) were purchased from Dupont (Boston, MA), sodium octyl sulfate from Eastman Kodak (Rochester, NY), and the scintillation fluid Betamax^R from ICN Biomedicals Inc. (Irvine, CA). The following compounds were from Sigma Chemical Co. (St. Louis, MO): ascorbate oxidase (E.C. 1.10.3.3.), 4-hydroxy-3-methoxyphenylglycol (MHPG) piperazinium salt, noradrenaline (NA) HCl, adrenaline (AD) free base, 5-hydroxytryptophan (5-HTP),5-hydroxyindole-3-acetic acid (5-HIAA), metanephrine (MTN) HCl, dopamine HCl, 4-hydroxy-3-methoxyphenylacetic acid (HVA), normetanephrine HCl, serotonin creatinine sulfate complex, 3-methoxytyramine HCl, 3,4-dihydroxyphenylacetic acid (DOPAC), (±)sulpiride base, haloperidol HCl, and Tris[hydroxymethyl]-aminomethane. The following drugs were purchased from Research Biochemicals Inc. (Natick, MA): S(+)- and R(-)-apomorphine HCl, S(+)bulbocapnine HCl, S(+)-

and R(-)butaclamol HCl, R(+)- and S (-)3PPP, R(+)SCH23390 HCl, S(-)SCH23388 HCl, (±)-, R(+)-and S(-)SKF38393 HCl, spiperone, fluphenazine HCl, and tyramine HCl. Domperidone and ketanserin were gifts from Janssen Pharmaceutica (Bersee, Belgium) and LY171555 or quinpirole HCl from Lilly Research Laboratories (Indianapolis, IN).

Dissection Procedures

The studies were performed with adult male New Zealand rabbits *Oryctolagus cuniculus* (1.5–2.0 kg, La Ferme Lapro Inc., Stukeley Sud, Québec) and male Sprague-Dawley rats (250–300 g, Charles-Rivers, St.-Constant, Québec). The animals were decapitated with a guillotine and their brains quickly (< 45 s) removed and placed on ice. A series of 1.0–1.5 mm thick sections (usually 7 or 8) were cut from each brain on a cold plate, and the cerebral cortex, hippocampus, and neostriatum were quickly dissected (Dewar et al., 1991; Reader et al., 1989b). Briefly, the cerebral cortex was subdivided into four areas according to the atlas of Zilles (1985) for the rat and the cytoarchitectonic maps of Rose (1931) and Fleischhauer, Zilles, and Schleicher, (1980) for the rabbit: cingulate, sensorimotor for the rabbit or somatosensory for the rat, piriform-entorhinal, and visual.

The hippocampal formation was removed from the 3–5 caudal most brain sections and separated into two parts: a dorsal portion, primarily composed of the Ammon's horn (areas CA1–CA5 in the rat and h1–h5 in rabbit) and dentate gyrus, or *Fascia dentata*, a ventral portion that included the ventral and dorsal *Subiculum* (Sub 1 and Sub 2). The neostriatum was isolated from the rostral most 3–5 sections. In the case of the rat, it was subdivided into rostral and caudal halves, using the anterior commissure as a landmark. In rabbit, the caudate nucleus, divided into lateral and medial halves, was separated from the putamen, care being taken to remove the white matter of the internal capsule.

Monoamine Assays

Tissue samples were placed in tubes containing 1960 µL cold monochloroacetic acid 0.1*M* to which were added 40 µL of ascorbate oxidase (1 mg/mL in 0.1*M* sodium monochloroacetate buffer, pH 3.3) to reduce the size of the "solvent front" (McKay et al., 1984; Sauvé and Reader, 1988). The samples were disrupted in a glass homogenizer with a Teflon^R pestle and the resulting homogenates centrifuged (12,500 rpm for 45 min at 4°C). The pellets were dissolved overnight in 1 mL of 1*N* NaOH for protein determinations (Lowry et al., 1951). The supernatants were used for determination of the compounds by reversed-phase high-performance liquid chromatography (HPLC) with ion-pairing and electrochemical detection following established procedures (Lakhdar-Ghazal et al., 1986; Reader and Grondin, 1987; Reader et al., 1988b). Briefly, the analytical column (250 × 4.6 mm) was packed with 5 µm particle-size RP_{18} and maintained at 30–32°C. The mobile phase was 0.1*M* sodium monochloroacetate pH 3.3–3.5 containing 750 mg/L Na_2EDTA, 450–500 mg/L sodium octyl sulfate, and 9 to 10% (v/v) methanol. This buffer was pumped at a flow rate of 0.6–0.8 mL/min (1200–1400 psi) and the working glassy carbon electrode was set at +750 mV against the Ag/AgCl reference. The separation procedure allowed the identification of MHPG, NA, DOPAC, AD, 5-HTP, 5-HIAA, MTN, DA, HVA, normetanephrine (NMN), 5-hydroxytryptamine (5-HT), and 3-methoxytyramine (3-MT) within 60 min. The coefficient of variation of the retention times throughout this study never exceeded 5%. Only the values obtained for DA and its main metabolites, DOPAC, HVA, and 3-MT, are reported here.

Radioligand Binding Assays

For the binding studies, samples from the total cerebral cortex and total neostriatum or from the discrete cortical, hippocampal and neostriatal regions were homogenized (Polytron, 15s) in 40–100 vol (w/v) of cold Tris-HCl buffer, pH 7.4 and

centrifuged (48,000*g*, 10 min, 4°C). After one wash by suspension and recentrifugation, the pellets were resuspended in 50 m*M* Tris-HCl buffer (pH 7.4) containing 120 m*M* NaCl, 5 m*M* KCl, 2 m*M* $CaCl_2$, and 1 m*M* $MgCl_2$ for the assay of D_1 receptors (Billard et al., 1984). The D_2 receptors were measured in Tris-Cl buffer (pH 7.4) with 120 m*M* NaCl and 5 m*M* KCl. The benzazepine [^{3}H]SCH23390 was used to label D_1 sites (Billard et al., 1984) and the benzamide [^{3}H]raclopride for the D_2 receptors (Köhler, Ogren, and Gawell, 1985). Unless otherwise indicated, the radioligands were added in 100 µL aliquots to tubes containing 200 µL of membrane preparations; the final assay vol were 500 µL.

For the competition experiments, the final assay vol were 1 mL. After incubating for 45 min at 25°C, binding was assessed by rapid filtration (< 5 s) over GF/C glass fiber filters, followed by two washes (< 10 s) with 5 mL cold buffer. Radioactivity was determined by liquid scintillation spectrometry in an LKB Rackbeta^R II counter (efficiency of converting counts per min to disintegrations per min was 52–62%). Specific binding was defined as the total binding minus the nonspecific counts obtained in the presence of either 30 µ*M* of the D_1 agonist (±)SKF38393 (Brière et al., 1987) or 300 µ*M* of the D_2 antagonist (±)sulpiride (Dewar et al., 1989). Protein concentrations determined (Lowry et al., 1951) in 100 µL aliquots of the membrane preparations were in the final incubations between 0.04–0.11 mg/mL for neostriatal and hippocampal samples and between 0.15–0.2 mg/mL for the cortical areas.

Data Analysis

The results are expressed as means ± SEM. The statistical significance of the differences between two samples was evaluated with the two-tailed Student's *t*-test. When more than one comparison was made, the significance of differences among samples was evaluated by one-way analysis of variance (T&ANOVAR; Barlow, 1983). Only probability values (p) smaller than 0.05 were considered significant. Model testing of the binding data was performed using LIGAND, a weighted nonlinear least-squares curve fitting program (McPherson, 1985; Munson and Rodbard, 1980), and the choice of the model that best fit the experimental data was achieved using the appropriate *F*-test.

Characterization of Dopamine D_1 Receptors

The binding characteristics of the novel benzazepine compound SCH23390 were studied using membrane preparations from rat and rabbit cerebral cortex and neostriatum (caudate-putamen). In all cases, the association kinetics of [^{3}H]SCH23390 were rapid, whereas the dissociation kinetics were extremely slow with only 40–60% of the binding displaced 2 h after the addition of either S(+)butaclamol or 30–50 vol of buffer (Reader et al., 1988a, 1989). The saturation curves revealed that [^{3}H]SCH23390 bound with high affinity in both tissues from the two species, with densities of 100–150 fmol/mg protein for cerebral cortex and 600–1000 fmol/mg protein for neostriatum (Fig. 1A). The affinity of [^{3}H]SCH-23390 for the D_1 receptor was comparable to that found in other mammalian species (Dawson et al., 1986; Briére et al., 1987; Madras et al., 1988), suggesting that this binding site has a similar high affinity within the different species studied.

The specificity of binding to the cortical D_1 receptor in both species was verified in competition experiments using a variety of dopaminergic agonists and antagonists, including enantiomer pairs. In the rabbit cerebral cortex (Reader et al., 1989), the rank order of potency for antagonists (Fig. 2) was determined to be R(+)SCH23390 > S(+)butaclamol > fluphenazine > S(-)SCH23388 > S(+)bulbocapnine > haloperidol > ketanserin > R(-)butaclamol >>> domperidone, spiperone, and (±)sulpiride. For agonists, it was R(+) SKF-38393 > R(-)apomorphine > S(+)apomorphine > S(-)3PP > (-)SKF38393 > DA > R(+)3PPP > LY171 555 > tyramine (Table 1). Less compounds were

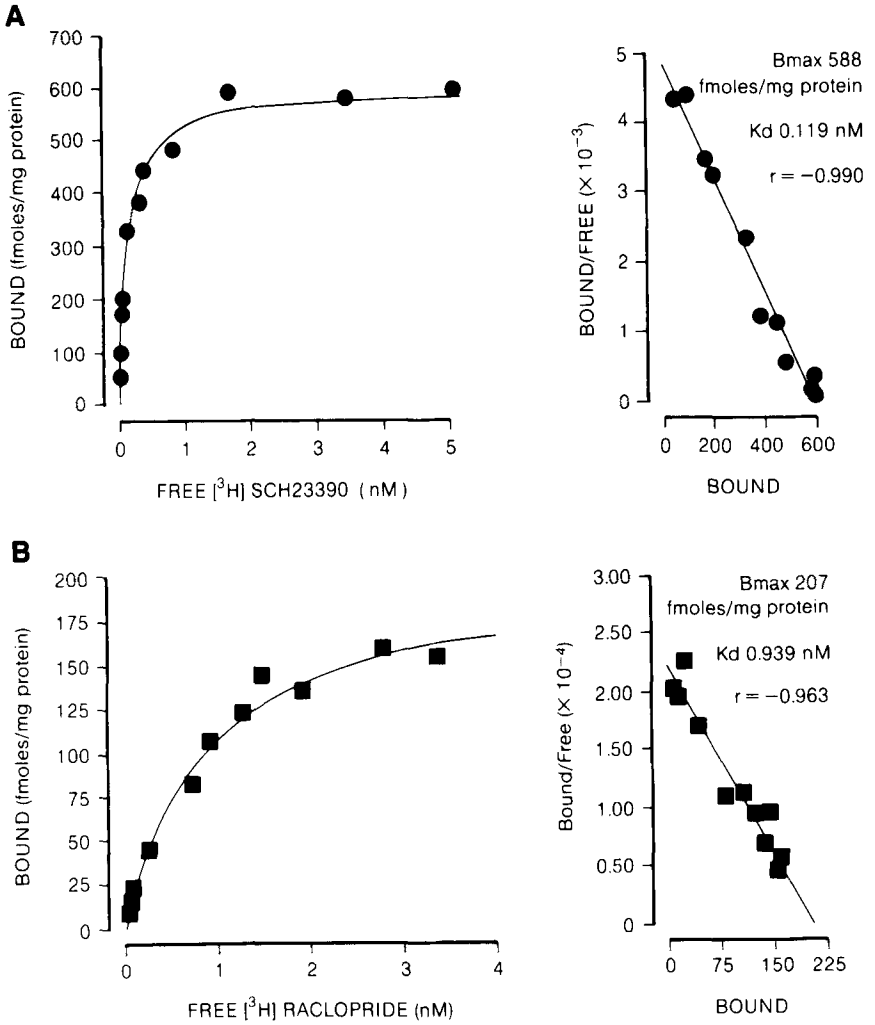

Fig. 1. Representative saturation curves of specific [^{3}H]SCH23390 and [^{3}H]raclopride binding to D_1 and D_2 receptors, respectively, in rabbit neostriatum. The experimental data points are the means of duplicate determinations, i.e., two tubes for total binding and two tubes for nonspecific counts in the presence of 30 μ*M* (±)SKF38393. The membrane preparations (200 μL) were added to tubes already containing 200 μL of buffer with or without unlabeled drug. The incubations were started by the addition of 10–12 concentrations of [^{3}H]SCH23390 (0.02–5 or 10 n*M*) or [^{3}H]raclopride (0.04–5 n*M*) and the binding (final volume 500 μL) proceeded for 45 min at 25°C as described in Materials and Methods. The bound/free vs bound analyses (Scatchard, 1949) are shown in the right panels, and *r* is the correlation coefficient (least-squares linear regression). The binding parameters for D_1 receptors **(A)** were a B_{max} of 588 fmol/mg protein with a K_D of 0.12 n*M* and for D_2 receptors **(B)**, a B_{max} of 207 fmol/mg protein with a K_D of 0.94 n*M*.

tested with membranes from rat cerebral cortex (Brière et al., 1987; Reader et al., 1988a), but the rank orders of potency were essentially the same. For the antagonists, it was R(+)SCH23390 > S(+) butaclamol > S(-)SCH23388 > S(+)bulbocapnine > spiperone > R(-)butaclamol, and for the agonists (±)SKF38393 > R(-)apomorphine > S(+) apomorphine > dopamine > tyramine (Table 1).

These rank orders of potency obtained with membrane preparations of both rabbit and rat cerebral cortex are consistent with the pharmacology of the dopaminergic D_1 site. Usually, all competition curves were better fitted to a one-site model with Hill coefficients around one (Fig. 2). In some cases, with rat tissue and only with agonist compounds, lower Hill coefficients

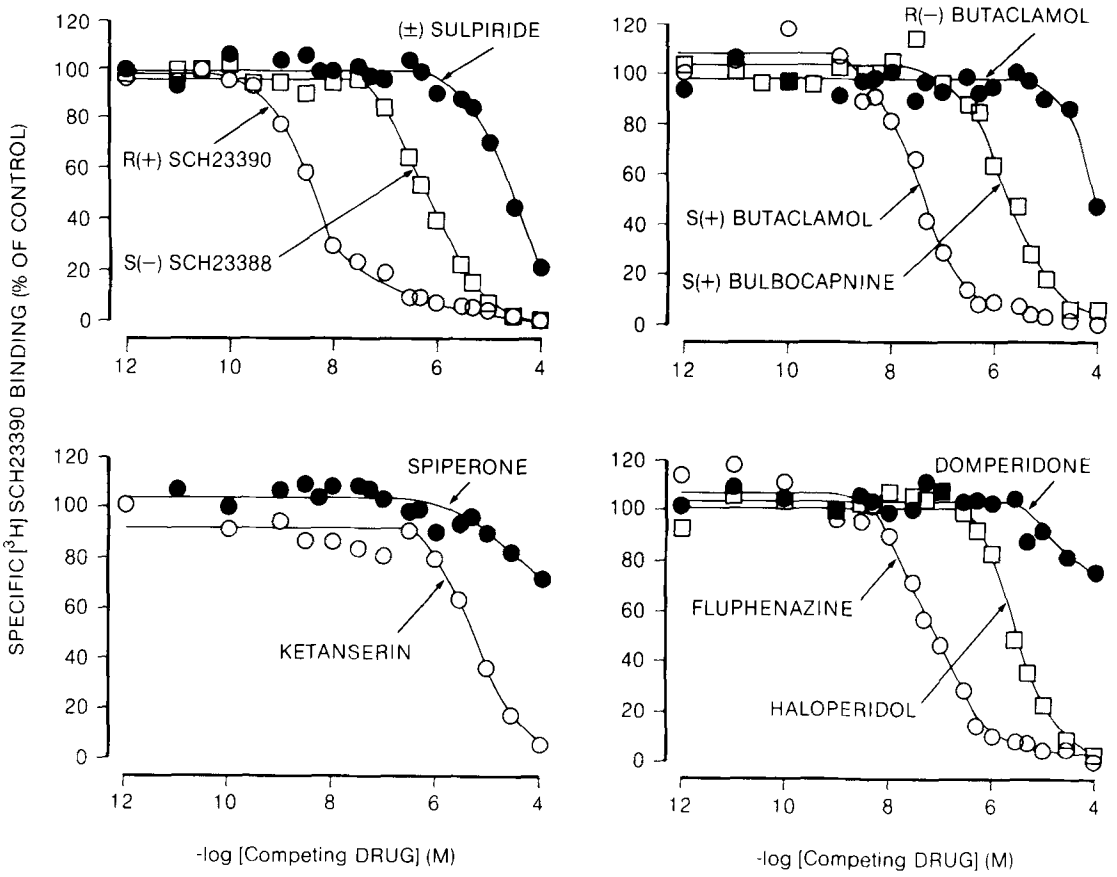

Fig. 2. Inhibition by dopaminergic antagonists of specific [^{3}H]SCH23390 binding to D_1 receptors of rabbit cerebral cortex. The membrane preparations (200 µL) were added to tubes already containing 700 µL of buffer with increasing concentrations (12–18) of the unlabeled drug. The incubations were started by the addition of 100 µL of the radioligand (final concentration 0.4 n*M*) and the binding proceeded for 45 min at 25°C, as described in the Methods section. Data points are the means of triplicate determinations from one representative experiment for each drug, and the theoretical curves were drawn by the iterative procedure INHIBITION, for a model with a single binding site (Parker and Waud, 1971; Barlow, 1983). Nonspecific binding was defined as the counts in the presence of 30–32 µ*M* (±)SKF38393.

(0.5–0.7) and a two-site model could be obtained, suggesting a certain heterogeneity of sites and/or a differential degree of coupling between the primary ligand recognition site and adenylate cyclase (Reader et al., 1988a; De Keyser et al., 1989). In this context, it has been reported that the D_1 receptor can exist in two agonist affinity states (Seeman and Grigoriadis, 1987). The stereoselectivity of the cortical [^{3}H]SCH23390 binding site of rat and rabbit was firmly documented by the use of enantiomer pairs of dopaminergic drugs: R(+)SCH23390 > S(-)SCH23388, S(+)butaclamol > R(-)butaclamol, R(+)SKF38393 > S(-)SKF 38393, R(-)apomorphine > S(+)apomorphine, and S(-) 3PPP > R(+)3PPP. Overall, these competition studies provided compelling evidence that [^{3}H]SCH23390 binds to DA D_1 receptors in the neostriatum and cerebral cortex of rat and rabbit.

Characterization of Dopamine D_2 Receptors

The binding properties of the substituted benzamide raclopride to DA D_2 receptors were studied with membrane preparations from rat and rabbit neostriatum. An analysis of the association kinetics suggested a single binding site,

Table 1
Inhibition Constants for [^{3}H]SCH23990 Binding to Membranes from Rabbit and Rat Cerebral Cortex

	Rabbit		Rat	
	K_i	n_H	K_i	n_H
Antagonists				
R(+)SCH23390	0.27 ± 0.03	0.76 ± 0.04	0.06 ± 0.02	0.64 ± 0.04
S(+)Butaclamol	17.1 ± 1.92	1.05 ± 0.07	9.60 ± 4.76	0.95 ± 0.12
Fluphenazine	38.4 ± 10.6	0.77 ± 0.06	—	—
S(−)SCH23388	180 ± 44.2	0.99 ± 0.02	210 ± 62.5	0.91 ± 0.03
S(+)Bulbocapnine	673 ± 91.1	0.95 ± 0.05	454 ± 188	0.97 ± 0.05
Haloperidol	1209 ± 70.6	1.02 ± 0.03	—	—
Ketanserin	8696 ± 2562	0.89 ± 0.15	—	—
R(−)Butaclamol	54819 ± 7350	1.07 ± 0.06	> 100000	—
Domperidone	> 100,000	ND	—	—
Spiperone	> 100,000	ND	8760 ± 2957	0.65 ± 0.14
(±)Sulpiride	> 100,000	ND	—	—
Agonists				
R(+)SKF38393	32.7 ± 4.2	0.89 ± 0.04	—	—
(±)SKF38393	—	—	77.7 ± 15.9	0.60 ± 0.20
R(−)Apomorphine	392 ± 78.4	1.02 ± 0.01	720 ± 17.5	0.96 ± 0.03
S(+)Apomorphine	428 ± 47.7	1.08 ± 0.02	1334 ± 336	0.97 ± 0.12
S(−)3PPP	5797 ± 1085	0.99 ± 0.06	—	—
S(−)SKF38393	7094 ± 1136	1.00 ± 0.06	—	—
Dopamine	13312 ± 2596	0.80 ± 0.07	8701 ± 1957	0.63 ± 0.07
R(+)3PPP	67768 ± 20842	0.83 ± 0.15	—	—
LY171555	> 100,000	ND	—	—
Tyramine	> 100,000	ND	> 100,000	ND

The inhibition of DA D_1 receptor binding was determined by incubating membrane preparations from rabbit and rat neostriatum with 0.25–0.50 n*M* [^{3}H]SCH23390 in the presence of 12–18 concentrations of the corresponding unlabeled drug. The values in nanomolar (n*M*) are the means ± SEM of three separate experiments, each performed in triplicate. The inhibition constants (K_i) were obtained by the method of Cheng and Prusoff (1973) from the IC_{50} values calculated by INHIBITION (Barlow 1983; Parker and Waud 1971). Nonspecific binding in all cases was defined by the counts obtained in the presence of 30–33 μ*M* (±)SKF38393. ND = not determined since the IC_{50} values were more than 200 μ*M*. (Data from Reader et al., 1988a, 1989).

but the data from the dissociation experiments were better described by a two-site model (Dewar et al., 1989), as has recently been reported for epidepride, another benzamide antagonist that labels D_2 receptors (Neve et al., 1990). Examination of saturation curves at equilibrium revealed a single class of binding sites in the neostriatum (Fig. 1B) from both species (rat B_{max} = 247 fmol/mg protein; rabbit B_{max} = 337 fmol/mg protein). Only a small number of binding sites could be detected in homogenates of total cerebral cortex (rabbit) with a B_{max} of 13 fmol/mg protein and a K_D (1.1 n*M*) that was not different from that found in the neostriatum (Dewar and Reader, 1989b).

The pharmacological selectivity in the neostriatum was examined by competition experiments with dopaminergic drugs. The rank of potency of agonists and antagonists to displace [^{3}H]raclopride binding revealed its selectivity for the DA D_2 receptor and was essentially the same for both species (Table 2). For the rabbit neostriatum, the rank order of potency was fluphenazine > S(+) butaclamol > spiperone > haloperidol > S(-) sulpiride > domperidone > R(+)SCH23390 > R(+) sulpiride > ketanserin > S(+)bulbocapnine > R(-)

Table 2
Inhibition Constants for [^{3}H]raclopride Binding to Membranes from Rabbit and Rat Neostriatum

	Rabbit		Rat	
	K_i	n_H	K_i	n_H
Antagonists				
Fluphenazine	0.24 ± 0.05	0.89 ± 0.09	0.64 ± 0.16	0.88 ± 0.04
S(+)Butaclamol	1.73 ± 0.56	0.79 ± 0.15	1.73 ± 0.65	0.79 ± 0.15
Spiperone	2.91 ± 0.13	0.96 ± 0.12	4.20 ± 1.31	0.69 ± 0.07
Haloperidol	13.90 ± 2.40	0.87 ± 0.02	8.64 ± 1.00	0.96 ± 0.05
S(–)Sulpiride	15.00 ± 1.01	0.77 ± 0.17	5.08 ± 1.01	0.62 ± 0.06
Domperidone	23.10 ± 2.67	0.81 ± 0.01	32.40 ± 14.8	0.71 ± 0.07
R(+)SCH23390	316.00 ± 51.8	0.82 ± 0.04	357.00 ± 33.6	0.86 ± 0.01
R(+)Sulpiride	909.00 ± 39.3	0.85 ± 0.17	838.00 ± 292	0.63 ± 0.07
Ketanserin	786.00 ± 143	0.54 ± 0.09	918.00 ± 98.3	0.62 ± 0.04
S(+)Bulbocapnine	1577.00 ± 36.8	0.94 ± 0.04	1470.00 ± 298	0.94 ± 0.04
R(–)Butaclamol	7499.00 ± 1684	0.93 ± 0.12	8463.00 ± 831	0.93 ± 0.12
	K_H	K_L	K_H	K_L
Agonists				
S(–)3PPP	42.40 ± 21.8	1344 ± 183	163.00 + 64.8[a]	ND
Dopamine	63.10 ± 14.8	5058 ± 967	27.60 ± 10.1	1956 ± 515
LY171555	69.40 ± 27.8	11,052 ± 4180	68.20 ± 19.6	4076 ± 1517
R(+)3PPP	80.60 ± 16.6	8944 ± 2796	190.00 ± 34.0	12,115 ± 3668
R(–)Apomorphine	85.60 ± 27.2	12,103 ± 5300	18.80 ± 1.8	3706 ± 758
S(+)Apomorphine	485.00 ± 83.1	48,600 ± 752	256.00 ± 68.7	30,600 ± 2140
(±)SKF389393[a]	5502.00 ± 586	ND	3626.00 ± 1236	ND
Tyramine[a]	> 50,000	ND	> 50000	

The inhibition of DA D_2 receptor binding was determined by incubating membrane preparations from rabbit and rat neostriatum with 1 n*M* [^{3}H]raclopride in the presence of 12–18 concentrations of the corresponding unlabeled drug. The values in n*M* are the means ±SEM of three separate experiments, each performed in triplicate. The inhibition constants (K_i) were obtained by the method of Cheng and Prusoff (1973) from the IC_{50} values calculated by INHIBITION (Barlow, 1983; Parker and Waud, 1971). n_H is the Hill coefficient. The competition parameters for agonists were obtained with a two-site model (LIGAND; Munson and Rodbard, 1980); K_H is the dissociation constant of the high-affinity site, and K_L the dissociation constant of the low-affinity site. Nonspecific binding in all cases was defined by the counts obtained in the presence of 300 μ*M* (±)sulpiride. [a]The inhibition curves for these agonist drugs could not be fitted to a two-site model and the K_i values are given. ND = not determined, since the IC_{50} values were above 200 μ*M*. (Modified from Dewar et al., 1989).

butaclamol. For rat neostriatum, the sequence was similar, only S(-)sulpiride was slightly more potent than haloperidol (Dewar et al., 1989).

All antagonist competition curves (Fig. 3) could be fitted to a single site, but inhibition by agonists was better described assuming a two-site model, with around 60% high-affinity and 40% low-affinity sites, in accord with previous studies conducted with other ligands showing that D_2 receptors exist in two agonist affinity states of approx equal proportion (De Lean, Kilpatrick, and Caron, 1982; Sibley, De Lean, and Creese, 1982; Seeman and Grigoriadis, 1987). For the rabbit, the rank order of potency for agonists was S(-)3PPP > DA > LY171555 > R(+)3PPP > R(-)apomorphine > S(+)apomorphine > (±)SKF38393 > tyramine, and for the rat R(-)apomorphine > dopamine > LY171555 > S(-)3PPP > R(+)3PPP > S (+) apomorphine > (±)SKF38393 > tyramine. The stereospecificity of binding in both species was demonstrated by the use of enantiomer pairs of antagonists, i.e.: S(+)butaclamol > R(-)butaclamol

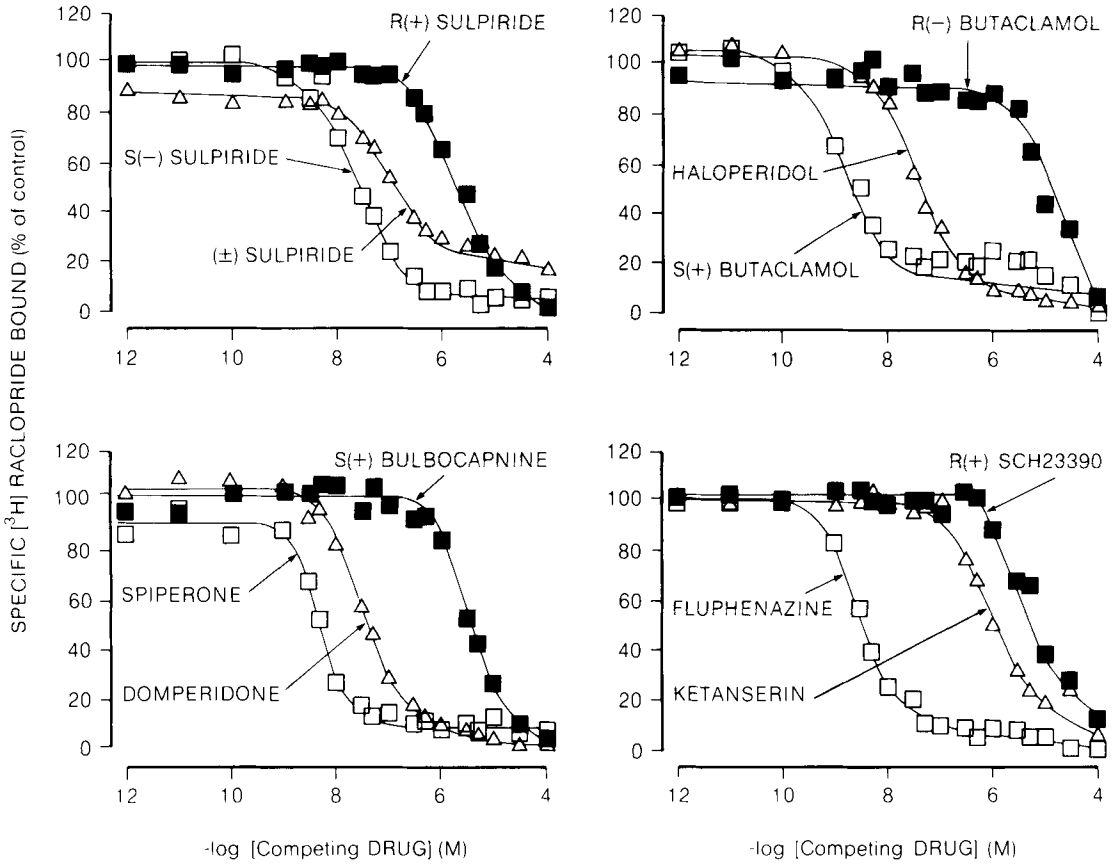

Fig. 3. Inhibition by dopaminergic antagonists of specific [^{3}H]raclopride binding to D_2 receptors of rabbit neostriatum. The membrane preparations (200 µL) were added to tubes already containing 700 µL of buffer with increasing concentrations (12–18) of the unlabeled drug. The incubations were started by the addition of 100 µL of the radioligand (final concentration 1.0 n*M*) and the binding proceeded for 45 min at 25°C, as described in the Methods section. Data points are the means of triplicate determinations from one representative experiment for each drug, and the theoretical curves were drawn by the iterative procedure INHIBITION, for a model with a single binding site (Parker and Waud, 1971; Barlow, 1983). Nonspecific binding was defined as the counts in the presence of 300 µ*M* (±)sulpiride.

and S(-)sulpiride > R(+)sulpiride, as well as agonists: S(-)3PPP > R(+)3PPP and R(-)apomorphine > S(+)apomorphine. These results validate the utilization of the novel benzamide [^{3}H]raclopride as a selective marker of dopamine D_2 receptors.

Sulfhydryl and Disulfide Groups

A combination of biochemical and radioligand binding techniques can be useful to investigate some of the regulatory properties and different affinity states of DA receptors. One of these approaches makes use of chemical reagents that react with -SH and -SS- of cysteine and cystine within the macromolecular receptor complex protein. Modification of these specific amino acids by reagents such as L-dithiothreitol (L-DTT) (Cleland, 1964) or *N*-ethylmaleimide (NEM) may result in alterations of receptor function, either at the primary ligand recognition site or at the level of the coupling to the second messenger system via G-proteins.

Such an approach has been used to characterize both nicotinic (Karlin and Bartels, 1966; Reader and De Robertis, 1974; Barrantes, 1980) and mus-

Table 3
Binding of [^{3}H]SCH23390 to Dopamine D_1 Receptors from Rat Cerebral Cortex and Neostriatum and of [^{3}H]raclopride to Dopamine D_2 Receptors from Rabbit Neostriatum in the Presence of L-DTT and NEM

	D_1 receptors				D_2 receptors	
	Rat cerebral cortex		Rat neostriatum		Rabbit neostriatum	
	B_{max}	K_D	B_{max}	K_D	B_{max}	K_D
Control	110 ± 4	0.38 ± 0.01	1040 ± 81	0.19 ± 0.005	272 ± 38	1.05 ± 0.08
30 μ*M* L-DTT	ND	ND	ND	ND	226 ± 27[g]	0.92 ± 0.15
1 m*M* L-DTT	ND	ND	ND	ND	96 ± 16[b]	0.66 ± 0.07
5 m*M* L-DTT	105 ± 3[e]	0.57 ± 0.04[b,f]	727 ± 24[b]	0.41 ± 0.10[a,d]	ND	ND
1 m*M* NEM	138 ± 5[b]	1.86 ± 0.03[c]	833 ± 51[a]	0.68 ± 0.03[b]	163 ± 26[a]	1.38 ± 0.26[d]
F values	18.980	748.192	7.807	15.270	7.550	3.438

The effects of disulfide and sulfhydryl reagents on specific D_1 and D_2 receptor binding were examined by treating membrane preparations for 30 min with buffer alone (control) or with buffer containing 30 μ*M*, 1 m*M* and 5 m*M* L-DTT, or 1 m*M* NEM. The membrane preparations were incubated with 10 concentrations of either [^{3}H]SCH23390 (0.01–10 n*M*; nonspecific binding defined in the presence of 32 μ*M* (±)SKF38393 for D_1 receptors) or [^{3}H]raclopride (0.05–10 n*M*; nonspecific binding defined by 300 μ*M* (±)sulpiride for D_2 receptors). Values are the means ±SEM of 4 to 5 independent experiments with separate membrane preparations, all performed in duplicate, i.e., two tubes for total binding and two tubes for nonspecific counts. The density of binding sites (B_{max}, maximum binding capacity) in femtomoles per miligram of protein (fmol/mg protein) and the equilibrium dissociation constant (K_D 25°C) in nanomolar (n*M*) were calculated by the nonlinear least-squares iterative curve fitting algorithm ENZFITTER (Leatherbarrow, 1987). The *F* values were obtained by a one-way analysis of variance (T&ANOVAR; Barlow, 1983), and the statistically significant differences determined were: [a]$P < 0.05$, [b]$P < 0.01$, and [c]$P < 0.001$, from controls; [d]$P < 0.05$, [e]$P < 0.01$, and [f]$P < 0.001$, between 1 or 5 m*M* L-DTT and 1 m*M* NEM, and [g]$P < 0.05$, between 30 μ*M* and 1 m*M* L-DTT.

carinic (Aronstam, Abood, and Hoss, 1978) cholinergic receptors, α-adrenergic receptors (Quennedy, Bockaert, and Ruout, 1984; Reader and Brière, 1985; Reader, Brière, and Grondin, 1986), β-adrenergic receptors (Stadel and Lefkowitz, 1979; Moxham and Malbon, 1985), DA D_1 receptors (Sidhu et al., 1986; Braestrup and Andersen, 1987; Dewar and Reader, 1989a), and DA D_2 sites (Suen, Stefanini, and Clement-Cormier, 1980; Freedman, Poat, and Woodruff, 1982; Kilpatrick, De Lean, and Caron, 1982; Sibley and Creese, 1983; Scheuhammer and Cherian, 1985). Furthermore, adenylate cyclase and G-proteins exhibit great sensitivity to inactivation by sulfhydryl alkylation with NEM (Malbon, George, and Moxham, 1987), although binding to the β-adrenergic receptor is seemingly spared (Moxham and Malbon, 1985). On the other hand, both α_1 and α_2 adrenoceptors are sensitive to disulfide reduction and sulfhydryl alkylation; the α_2 sites being more sensitive to NEM, a treatment that leads to important decreases in the affinity for [^{3}H]idazoxan (Reader, Briere, and Grondin, 1986).

Sulfhydryl and Disulfide Groups of D_1 Receptors

It also has been shown that [^{3}H]SCH23390 binding to D_1 receptors can be modified by reagents that react with -SS- and -SH groups (Sidhu et al., 1986; Braestrup and Andersen, 1987). In fact, in the rat neostriatum and cerebral cortex, D_1 receptors labeled with [^{3}H]SCH23390 require the integrity of -SH groups to maintain a high affinity of the receptor protein, whereas -SS- bonds are essential to keep the maximum binding capacity (Dewar and Reader, 1989a). Treatment of cortical and neostriatal membranes with L-DTT or NEM produced dose-dependent decreases of specific [^{3}H]SCH23390 binding (Table 3). These changes were not reversible after up to two washes but could be prevented in part if the treatments were performed in the presence

of DA. In fact, DA protected neostriatal and cortical [^{3}H]SCH23390 binding sites from inactivation by L-DTT, whereas the NEM-mediated alkylation of D_1 receptor in these two tissue preparations remained unaffected. Partial protection against L-DTT was also possible with both (+) and (-) enantiomers of the selective agonist SKF38393; however, neither enantiomer protected [^{3}H]SCH 23390 binding sites from alkylation by NEM (Dewar and Reader, 1989a).

Sulfhydryl and Disulfide Groups of D_2 Receptors

Receptor binding studies were performed in rabbit neostriatum using [^{3}H]raclopride (Table 3). Treatment of the membrane preparations with the reducing agent L-DTT as well as with the alkylating compound NEM produced dose-dependent decreases of specific [^{3}H]raclopride binding; the IC_{50} values were 3.1 and 1.2 m*M*, respectively. Saturation experiments showed that the reduction of -SS- bonds by 1 m*M* L-DTT decreased the number of binding sites with only a slight increase in the affinity. On the other hand, alkylation of -SH groups by NEM (1 m*M*) decreased both receptor number and affinity. Competition curves with the physiological substrate DA and with the dopaminergic antagonist (+)butaclamol were used to examine the properties of the binding sites.

The IC_{50} values for (+)butaclamol in control and in L-DTT- and NEM-treated membranes were between 3.4 and 4.8 n*M*, with Hill coefficients (n_H) of 1, indicating that the remaining binding sites conserved a high affinity for antagonist binding. In the case of DA, the curves were shallow (n_H = 0.45–0.64), and both compounds increased the IC_{50} from 0.7 μ*M* (control) to 8 μ*M* and 11 μ*M* for L-DTT and NEM, respectively. Iterative analysis revealed that L-DTT produced a very important (> 60%) decrease in the number of high-affinity (R_H)-binding sites but only a slight increase in the affinity of the low-affinity (K_L) site. After NEM, there was a decrease in both the number (R_H) and affinity (K_H) of the high-affinity binding sites. These results demonstrate the participation of -SS- and -SH groups in the agonist conformation of the primary ligand recognition site of the DA D_2 receptor. Alternatively, -SS- and -SH groups could be related to the coupling of the primary ligand recognition protein with adenylate cyclase by means of an inhibitory type of G-protein.

Ionic Regulation of Cortical and Neostriatal DA Receptors

Previous electrophysiological studies, using the iontophoretic technique to record and apply dopaminergic compounds, GABA, acetylcholine, and LiCl on neurons in the primary visual cortex of the rat, have documented interactions between lithium (Li^+) and cortical DA receptors (Gottberg, Montreuil, and Reader, 1988). The main responses to DA or the D_1 agonist (±)SKF38393 on spontaneously active or visually driven units was a prolonged decrease in neuronal firing and a reduction in the responsiveness to pulses of acetylcholine. The D_1 antagonist SCH23390, applied iontophoretically or intravenously, blocked or attenuated the inhibitory responses to both DA and (±)SKF38393. The D_2 agonist LY171555 (quinpirole) either produced only slight excitations or had no effects on neuronal firing. The concomitant application of Li^+ blocked the inhibitory responses to DA and (±)SKF38393 but did not modify the responsiveness to LY171555.

In addition, the dopamine- and (±)SKF38393-induced decreases in responsiveness to acetylcholine also were suppressed by Li^+. These effects were on dopaminergic mechanisms, since the excitatory responses to acetylcholine alone as well as the inhibition caused by GABA were unchanged by the application of Li^+. The results imply that the modifications in sensitivity to dopaminergic agents induced by Li^+ are mediated by DA D_1 receptors and could be related to inactivation of adenylate cyclase, i.e., the second messenger system. Another possibility was that Li^+ could be interfering with the receptor com-

Table 4
Monovalent Cation Regulation of Dopamine D_1 Receptors in Rat Cerebral Cortex and Neostriatum

	NaCl	LiCl	Sucrose	F
Cerebral cortex				
		Saturation		
B_{max}	108.00 ± 4.00[b]	99.00 ± 6.00[f]	185.00 ± 13.00	30.330
K_D	0.25 ± 0.03[b]	0.32 ± 0.03[d]	0.55 ± 0.09	7.465
n_H	0.86 ± 0.07	0.79 ± 0.06	0.88 ± 0.05	0.609
		Competition with dopamine		
IC_{50}	7471 ± 709[b]	7452 ± 1743[e]	1166 ± 215	11.049
Neostriatum				
		Saturation		
B_{max}	1074.00 ± 174	1054 ± 194	1084 ± 13.00	0.004
K_D	0.15 ± 0.07[a,b]	0.23 ± 0.01	0.25 ± 0.02	10.867
n_H	0.94 ± 0.01	0.89 ± 0.02	0.96 ± 0.03	2.634
		Competition with dopamine (one-site analysis)		
IC_{50}	6222 ± 1071[b]	6882 ± 1157[e]	1826 ± 509	8.256
		Competition with dopamine (two-site analysis)		
K_H	510.00 ± 180	826.00 ± 321	500.00 ± 124	0.546
K_L	10,475 ± 1699[b]	11,165 ± 2327[d]	4475 ± 1087	4.283
$\%R_H$	26.10 ± 2.1[c]	26.70 ± 5.2[f]	75.10 ± 5.9	35.455
$\%R_L$	73.90 ± 2.1[c]	73.30 ± 5.2[f]	24.90 ± 5.9	35.487

The results are the means ±SEM of four saturation experiments and three to four competition curves, all performed in duplicate or triplicate, and with separate membrane preparations. The B_{max} values are in femtomoles per milligram of protein, and the dissociation and inhibition constants (K_D and IC_{50}) are in nanomolar (n*M*); n_H is the Hill coefficient. K_H and K_L are the dissociation constants in nanomolar of the high- and low-affinity sites, respectively; $\%R_H$ and $\%R_L$ are the percentage of sites at high- and low-affinity. All buffers were made of Tris-Cl 10 m*M* (pH 7.4) with 1 m*M* $MgCl_2$, and contained either NaCl 120 m*M*, LiCl 120 m*M* or sucrose. The *F* values were obtained by a one-way analysis of variance (T&ANOVAR; Barlow, 1983) and the significant differences between the buffers had the following probabilities: [a] $p < 0.01$, between NaCl and LiCl; [b] $p < 0.01$, [c] $p < 0.001$, between NaCl and sucrose; [d] $p < 0.05$, [e] $p < 0.01$, and [f] $p < 0.001$, between LiCl and sucrose.

plex, i.e., by occluding or blocking the primary ligand recognition site. Therefore, a series of in vitro studies of ionic regulation on the binding parameters of DA receptors were conducted with membrane preparations from rat cerebral cortex and neostriatum (D_1 receptors labeled with [^{3}H]SCH23390) and from rabbit neostriatum (D_2 receptors labeled with [^{3}H]raclopride).

In Vitro Effects on D_1 Receptors

Saturation binding isotherms of [^{3}H]SCH23390 for rat cerebral cortex and neostriatum revealed a significantly greater affinity when either 120 m*M* sodium (Na^+) or Li^+ was added to the incubation buffer, made of Tris-HCl 10 m*M* containing 1 m*M* $MgCl_2$ (Gottberg et al., 1989). In the cerebral cortex but not the neostriatum, Na^+ increased the affinity of [^{3}H]SCH23390 binding to a significantly greater extent than Li^+ (Table 4). Furthermore, there was an almost 50% loss of cortical [^{3}H]SCH23390 binding sites in the presence of either Na^+ or Li^+. The higher B_{max} and K_D values of [^{3}H]SCH23390 binding in the absence of Na^+ or Li^+ could be explained by loss of selectivity of

this ligand. In fact, previous studies had demonstrated that [^{3}H]SCH23390 labels both the 5-HT_2 and 5-HT_{1C} receptors under appropriate conditions (Bischoff et al., 1986; Hoyer and Karf, 1988). The lack of specificity of [^{3}H]SCH23390 in the absence of Na^+ was substantiated by competition curves that revealed a residual binding that could not be displaced even by high concentrations of DA (Gottberg et al., 1989). This finding is in line with the reports indicating that D_1 receptors in cerebral cortex may be overestimated with [^{3}H]SCH23390 because this D_1 antagonist displaces the serotonin antagonist [^{3}H]ketanserin from 5-HT_2 sites with a K_i of 7 n*M*; conversely, unlabeled ketanserin can compete with [^{3}H]SCH23390. In fact, in the absence of Na^+, [^{3}H]SCH23390 binding to cerebral cortex is inhibited by ketanserin following a two-site model (Hess et al., 1986).

The saturation curves with membrane preparations from the neostriatum revealed no changes in receptor densities, but the affinity was greater in the presence of Na^+ (Table 4). In addition, the IC_{50} values for DA competitions (one-site analysis) were higher in both Na^+ and Li^+ than in sucrose. When these curves were analyzed for a two-site model with LIGAND (McPherson, 1985; Munson and Rodbard, 1980), there were no changes in the affinity of the high-affinity site (K_H), but the affinity of the low-affinity site (K_L) decreased more than twofold in the presence of Na^+ and Li^+, as compared to sucrose. Interestingly, there was a shift in the proportions of high- (% R_H) and low-affinity (% R_L) sites in the presence of both cations as compared to the values obtained in their absence (sucrose).

In Vitro Effects on D_2 Receptors

The addition of Na^+ to the incubation buffer enhanced the binding of [^{3}H]raclopride to D_2 receptors in the rabbit neostriatum in a concentration-dependent manner (Reader et al., 1990). A similar increase of specific [^{3}H]raclopride binding by Na^+ in monkey cerebral cortex has also been shown (Lidow et al., 1989). In our studies, the substitution of Li^+ for Na^+ produced a similar elevation in binding, although Li^+ was only around 50% as effective. The ability of these ions to augment [^{3}H]raclopride binding appears to be related primarily to an alteration in the affinity of the ligand, since saturation binding isotherms for [^{3}H]raclopride to rabbit neostriatum revealed both Na^+ and Li^+ significantly increased the affinity (as shown by a decrease in the K_D values; Table 5). The affinity change occurring in the presence of Li^+ was around two times smaller than that found with Na^+.

In the presence of Na^+ or Li^+, the B_{max} values of [^{3}H]raclopride binding were significantly higher than in the control buffer; however, since binding in the absence of ions was not saturable at the highest concentration (about 10 n*M*) of radioligand employed (Fig. 4), the estimate of B_{max} obtained in sucrose buffer may not reflect accurately the real density of D_2 receptors (Fig. 5). Interestingly, the guanine nucleotide derivative 5'-guanylylimidodiphosphate, or Gpp(NH)p, reduced the potency of DA to compete with [^{3}H]raclopride binding in the presence and absence of cations; however, this effect of Gpp(NH)p was a shift of the D_2 receptors from a high to a lower affinity state (Table 5). This conversion was incomplete; but, it has been reported that the capability of guanine nucleotides to modulate agonist binding to D_2 receptors may be dependent on their anatomical localization (De Keyser et al., 1985). In contrast to the classical DA D_2 antagonists, Na^+ has been found to be an absolute requirement for binding of the benzamide series of D_2 antagonists (Stefanini et al., 1980; Freedman, Poat, and Woodruff, 1982; Hall, Köhler, and Gawell, 1985; Niznik et al., 1985). It has been proposed that the benzamides bind to the D_2 receptor in a two-stepped manner involving a sodium-dependent isomerization of the site that increases the affinity of the receptors for the radioligand (Neve et al., 1990). In the absence of Na^+, the isomerization of the D_2 receptor would not occur so that benzamides would only bind with low affinity.

The binding of agonists to the D_1 and D_2 receptors is known to be regulated by Na^+

Table 5
Monovalent Cation Regulation of Dopamine D_2 Receptors in Rat Neostriatum

	NaCl	LiCl	Sucrose	*F*
		Saturation		
B_{max}	448.00 ± 29.7[e]	400.00 ± 32.4[f]	254.00 ± 37.1	9.092
K_D	0.99 ± 0.13[a,e]	2.37 ± 0.24[f]	6.41 ± 0.97	25.911
		Competition (one-site analysis)		
(+)Butaclamol				
IC_{50}	9.93 ± 1.58	6.29 ± 0.27	9.07 ± 1.93	1.725
n_H	1.08 ± 0.03[b,c]	1.35 ± 0.06[g]	0.90 ± 0.02	31.408
Dopamine				
IC_{50}	586.60 ± 31.7[a,d]	352.60 ± 45.4	263.60 ± 73.5	9.861
n_H	0.54 ± 0.03	0.50 ± 0.02	0.56 ± 0.05	0.737
		Effects of guanine nucleotide on dopamine competition (two-site analysis)		
Control K_H	137.10 ± 37.8[a,c]	47.30 ± 4.2	24.90 ± 5.2	7.178
Gpp(NH)p K_H	138.90 ± 69.1	48.70 ± 15.3	50.00 ± 28.4	1.379
Control K_L	5612.00 ± 1330[a,c]	1794.00 ± 603	1432.00 ± 630	6.361
Gpp(NH)p K_L	5312.00 ± 826[a,d]	2377.00 ± 217	1176.00 ± 660	11.658
Control $\%R_H$	62.00 ± 3.8	53.60 ± 8.2	59.10 ± 8.6	0.351
Gpp(NH)p $\%R_H$	34.70 ± 1.2[i]	23.90 ± 2.5[h]	46.20 ± 13.4	1.992
Control $\%R_L$	38.00 ± 3.8	46.40 ± 8.2	40.90 ± 8.6	0.351
Gpp(NH)p $\%R_{Lc}$	65.30 ± 1.2[i]	76.10 ± 2.5[h]	53.80 ± 13.4	1.992

The results are the means ±SEM of 9 to 10 saturation experiments and three to four competition curves, all performed in duplicate or triplicate, and with separate membrane preparations. The B_{max} values are in femtomoles per milligram of protein, while the equilibrium dissociation constants (K_D), the inhibition constants (IC_{50}), the dissociation constants of the high-affinity site (K_H) and the dissociation constants of the low-affinity site (K_L) are in nanomolar (n*M*). $\%R_H$ and $\%R_L$ are the percentage of sites at high and low affinity. The *F* values were obtained by a one-way analysis of variance (T&ANOVAR; Barlow, 1983), and the significant differences between the buffers had the following probabilities: [a]$P < 0.05$ and [b]$P < 0.01$, between NaCl and LiCl; [c]$p < 0.05$, [d]$p < 0.01$, and [e]$p < 0.001$, between NaCl and sucrose; [f]$p < 0.01$ and [g]$p < 0.001$ between LiCl and sucrose. Statistical significance between the values obtained in the absence (control) and presence of the guanine nucleotide Gpp(NH)p was determined by Student's *t*-test; [h]$p < 0.05$ and [i]$p < 0.01$.

(Makman, Dvorkin, and Klein, 1982; Hamblin and Creese, 1982; Neve, 1991). In accordance with these data, the affinity of DA to compete with [^{3}H]SCH23390 and [^{3}H]raclopride binding was decreased in the presence of Na^+ or Li^+. It has been reported that in the presence of sodium, D_1 receptors convert from a state having a high affinity for DA (D_1^{HIGH}) to one having a lower affinity (D_1^{LOW}). The findings that Na^+ and Li^+ produce a significant loss of the high-affinity state of the D_1 receptor with a small but still significant increase in the low-affinity site is consistent with this hypothesis. In addition to this conversion from D_1^{HIGH} to D_1^{LOW}, the presence of Na^+ or Li^+ in the incubation buffer also resulted in a significant decrease in the affinity of dopamine for the low-affinity site (Table 4).

In contrast to the D_1 receptor, the alteration in the affinity of the D_2 receptor for DA appeared to be related to a loss of affinity for both the D_2^{HIGH} and D_2^{LOW} sites (Table 5) and not to the conversion of the two states of the D_2 receptor as reported for classical D_2 antagonists (Grigoriadis and Seeman, 1985,1986). The effects of Na^+ and Li^+ on the binding properties of [^{3}H]SCH23390 and [^{3}H]raclopride were qualitatively similar. In

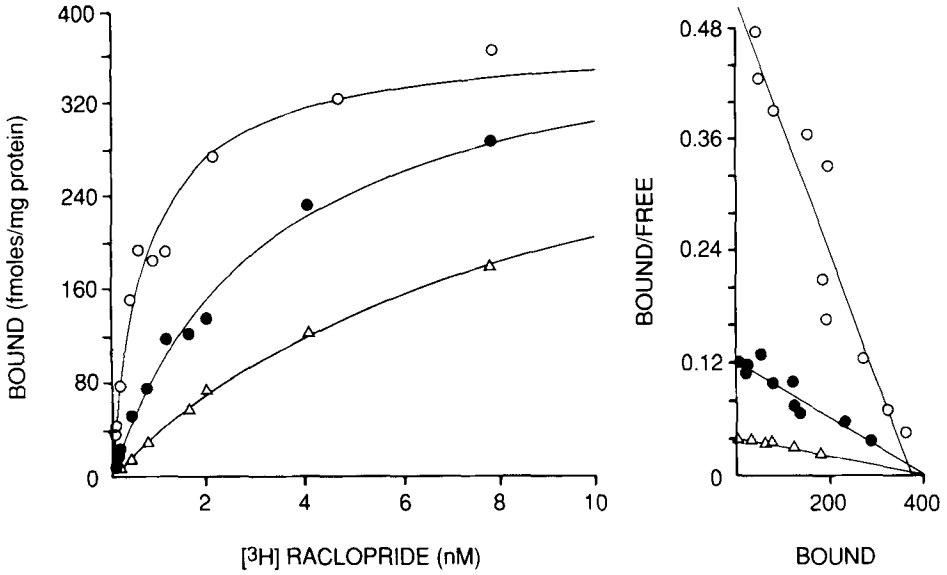

Fig. 4. Saturation curves of specific [^{3}H]raclopride binding to D_2 receptors of rabbit neostriatum in the presence of 120 m*M* of either NaCl, LiCl or sucrose. The data points are means of duplicate determinations, i.e., two tubes for total binding and two tubes for nonspecific counts in the presence of 300 μ*M* (±)sulpiride. The membrane preparations (300 μL) were added to tubes already containing 600 μL of buffer with or without (±)sulpiride. The incubations were started by addition of 100 μL aliquots of the radioligand (10 concentrations; 0.05–10 n*M*), and the binding proceeded for 45 min at 25°C, as described in the Methods section. The curves were constructed by the non-linear iterative procedure ENZFITTER (Leatherbarrow, 1987) for a model with a single binding site and the bound/free vs bound analyses (Scatchard, 1949) are shown in the right panel. ○, NaCl; ●, LiCl; Δ, Sucrose.

addition, the influence of Na^+ and Li^+ on agonist binding to the D_1 and D_2 receptors suggests that these cations may act at the same site of the ligand receptor complex for both ligands. In the case of [^{3}H]SCH23390 binding to D_1 receptors of cerebral cortex and neostriatum, both cations produced identical effects with only small differences being apparent in the K_D value for [^{3}H]SCH23390, and the changes in the affinity of DA binding to the D_1 receptor were almost identical in the presence of Na^+ and Li^+, suggesting that the latter can completely replace Na^+ in this system. In contrast, binding of [^{3}H]raclopride in a medium containing Li^+ was around 50% of that found in the presence of Na^+, indicating that for D_2 receptors labeled with benzamides, Li^+ can only partially replace Na^+. This finding is consistent with binding of [^{3}H]sulpiride to rat striatal membranes, where substitution of Na^+ by Li^+ resulted in a reduction in specific binding to around 55% (Theodorou et al., 1980).

Distribution and Postnatal Development

The densities of DA D_1 and D_2 receptors were measured using [^{3}H]SCH23390 and [^{3}H]raclopride, respectively, in the rabbit and rat cortical areas, hippocampus, and neostriatum (Diop et al., 1988; Dewar and Reader, 1989b). Dopamine and its metabolites DOPAC, HVA, and 3-MT were assayed by HPLC with electrochemical detection (Table 6). For both species, the distributions of [^{3}H]SCH23390 and [^{3}H]raclopride binding were heterogenous with the greatest densities in the neostriatum. In this region, D_1 receptors had B_{max} values of 700–900 and 570–1120

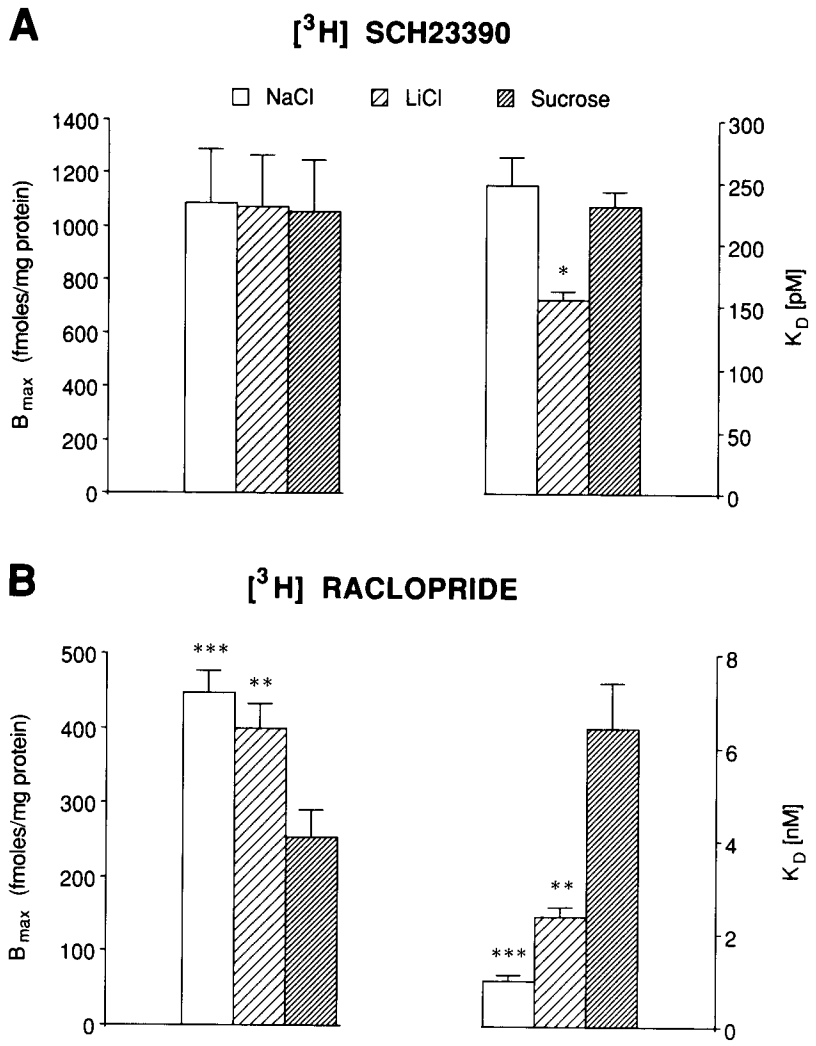

Fig. 5. Effects of the ionic composition of the incubation buffer on specific binding to DA D_1 and D_2 receptors of rabbit neostriatum. The values are the means ±SEM of four independent saturation curves for each buffer and each radioligand. **A**: For D_1 receptors labeled with [^{3}H]SCH 23390, there were no changes in receptor number but an increase in the affinity when the buffer contained NaCl. **B**: In the absence of cations, binding of [^{3}H]raclopride to D_2 receptors was very low because of a very low affinity; and because of this loss of saturability (Fig. 4), the B_{max} values could have been underestimated. In addition, the affinity was higher in NaCl than in LiCl.

fmol/mg protein for rabbit and rat, respectively. In the case of D_2 receptors, receptor densities were lower, i.e., 235–335 fmol/mg protein in the rabbit and 180–360 fmol/mg protein in the rat (Table 7). The concentrations of DA and its metabolites also were highest in this structure. Regions with low DA content, i.e., cortex and hippocampus, had lower densities of [^{3}H]SCH23390 and [^{3}H]raclopride binding.

In the rabbit and rat cerebral cortex, the densities of D_1 receptors ranged from 100 to 260 fmol/mg protein, but lower amounts were measured in the hippocampus (Table 7). Dopamine D_2 receptors in cortical regions known to possess a distinct dopaminergic innervation (piriform-entorhinal and cingulate cortex) ranged between 9 and 22 fmol/mg protein. The [^{3}H]raclopride binding sites (< 12 fmol/mg protein) were also

Table 6
Regional Distribution of Dopamine and Metabolites in Rabbit and Rat

Region	Dopamine, ng/mg p.	DOPAC, ng/mg p.	HVA, ng/mg p.	3-MT, ng/mg p.
		Rabbit		
Cin	0.36 ± 0.05	0.45 ± 0.03	7.89 ± 0.97	0.04 ± 0.02
Ss	0.11 ± 0.06	0.26 ± 0.07	4.71 ± 0.46	0.10 ± 0.02
Vis	0.04 ± 0.01	0.24 ± 0.08	0.66 ± 0.03	0.04 ± 0.01
PiEn	1.97 ± 0.17	1.33 ± 0.18	8.76 ± 1.07	0.29 ± 0.13
dH	0.18 ± 0.07	0.21 ± 0.04	1.84 ± 0.21	0.02 ± 0.01
vH	0.13 ± 0.03	0.24 ± 0.04	2.16 ± 0.17	0.04 ± 0.03
lNS	68.07 ± 7.34	27.15 ± 1.93	86.12 ± 6.83	14.25 ± 1.50
mNS	83.34 ± 9.11	35.69 ± 3.01	82.57 ± 6.60	14.81 ± 2.72
Pu	71.55 ± 8.29	28.24 ± 3.72	113.00 ± 10.2	11.84 ± 1.87
		Rat		
Cin	0.65 ± 0.11	0.82 ± 0.12	0.54 ± 0.09	0.04 ± 0.01
Ss	0.04 ± 0.01	0.14 ± 0.02	0.04 ± 0.01	0.03 ± 0.01
Vis	0.02 ± 0.01	0.07 ± 0.01	0.02 ± 0.01	0.03 ± 0.01
PiEn	1.49 ± 0.30	1.37 ± 0.11	1.07 ± 0.13	0.05 ± 0.01
H	0.02 ± 0.01	0.15 ± 0.01	0.10 ± 0.02	0.02 ± 0.01
NS	81.35 ± 3.41	37.16 ± 1.61	12.06 ± 0.30	9.69 ± 0.55
rNS	99.70 ± 8.80	65.60 ± 9.70	14.30 ± 1.00	6.60 ± 1.00
cNS	46.30 ± 9.30	34.70 ± 7.20	7.40 ± 1.00	4.20 ± 0.70

The results are the means ±SEM (n = 6–12) in nanograms per milligram of protein (ng/mg p.). The cortical regions are anterior cingulate (Cin), sensorimotor (rabbit) or primary hindlimb somatosensory (Ss), primary visual (Vis), entorhinal and piriform (PiEn). The other structures are the hippocampus (H) for the rat or the dorsal (dH) and ventral (vH) divisions for the rabbit, the total neostriatum (NS), or the rostral (rNS) and caudal (cNS) divisions for the rat, or the lateral (lNS) and medial (mNS) neostriatum and the putamen (Pu) for the rabbit. The metabolites are 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), and 3-methoxytyramine (3-MT). (Modified from Dewar and Reader, 1989b; Dewar et al., 1990; Diop et al., 1988; Reader and Dewar, 1989; and Reader et al., 1988a).

detectable in the dorsal and ventral hippocampus as well as in the somatosensory and visual cortices. In both species, there was a very good correlation between receptor densities and endogenous DA content.

For the rabbit, the correlation between DA and D_1 receptors had a regression coefficient (r) of 0.994 ($p < 0.001$), and between DA and D_2 receptors an r of 0.962 ($p < 0.001$). Similarly, for the rat, the correlation coefficients between DA and receptors were 0.990 ($p < 0.001$) and 0.991 ($p < 0.001$) for D_1 and D_2 binding sites, respectively. The heterogeneities in the distribution of D_1 and D_2 receptors can be related to the dopaminergic innervation and the turnover estimated by the ratios between endogenous DA and its metabolites. The presence of cortical DA receptors, even in low amounts (Lidow et al., 1989), warrants additional interest in their study as potential targets for neuroleptic drugs (Seeman et al., 1976) and to determine their possible role in psychiatric diseases such as schizophrenia. It has also been recently shown that very low levels of the DA D_{2A} receptor gene have been measured in cortical areas of human brain (Gandelman et al., 1991).

The developmental binding parameters of DA receptors were examined at 15, 30, and 90 d

Table 7
Distribution of Dopamine D_1 and D_2 Receptors, Their Ratios, Endogenous Dopamine, and Turnover Index

Region	D_1 receptors, fmol/mg p.	D_2 receptors, fmol/mg p.	D_1/D_2	DA, pmol/mg p.	TI
			Rabbit		
Cin	152 ± 12	9.3 ± 1.3	16.34	2.35 ± 0.33	29.92
Ss	141 ±14	7.0 ± 1.4	21.71	0.72 ± 0.39	54.19
Vis	173 ± 18	12.3 ± 1.7	14.06	0.26 ± 0.06	34.05
PiEn	152 ± 7	17.0 ± 4.3	8.94	12.99 ± 1.11	5.33
dH	135 ± 16	5.6 ± 1.3	24.10	1.18 ± 0.46	68.27
vH	84 ± 11	9.6 ± 1.2	8.75	0.85 ± 0.20	54.42
lNS	820 ± 101	335.0 ± 36.7	2.45	444.90 ± 47.97	1.93
mNS	926 ± 143	252.0 ± 9.4	3.67	544.71 ± 59.54	1.61
Pu	731 ± 83	235.0 ± 21.9	3.11	467.65 ± 54.18	2.20
			Rat		
Cin	192 ± 28	22.2 ± 2.1	8.65	4.24 ± 0.72	2.16
Ss	104 ± 6	10.0 ± 2.4	10.40	0.24 ± 0.05	5.69
Vis	110 ± 12	11.4 ± 2.2	9.65	0.10 ± 0.02	7.25
PiEn	260 ± 38	14.7 ± 2.1	17.69	9.73 ± 1.96	1.67
dH	ND	13.4 ± 2.6	—	—	—
vH	63 ± 4	9.6 ± 1.6	6.56	0.16 ± 0.30	11.33
NS	1005 ± 51	364.0 ± 20.3	2.76	531.06 ± 22.26	0.72
rNS	1117 ± 137	358.0 ± 10.0	3.12	651.64 ± 57.52	0.87
cNS	573 ± 73	180.0 ± 12.0	3.18	302.61 ± 60.78	1.00

The results are the means ±SEM ($n = 6$). The cortical regions are anterior cingulate (Cin), sensorimotor (rabbit) or primary hindlimb somatosensory (Ss), primary visual (Vis), entorhinal and piriform (PiEn). The other structures are the the dorsal (dH) and ventral (vH) divisions of the hippocampus, the total neostriatum (NS), or the rostral (rNS) and caudal (cNS) divisions for the rat, or the lateral (INS) and medial (mNS) neostriatum and the putamen (Pu) for the rabbit. The distribution of DA receptors was conducted by incubating membrane preparations from the dissected regions at fixed ligand concentrations; 5 n*M* of [^{3}H]SCH23390 for D_1 receptors and 4 n*M* of [^{3}H]raclopride for D_1 receptors. Nonspecific binding was defined by 30–35 μ*M* of (±)SKF38393 or 300 μ*M* (±)sulpiride for D_1 and D_2 receptors, respectively. The B_{max} values were obtained from the specific bound values calculated by the following formula: B_{max} = Bound x $(K_D + L)/L$, where L is the radioligand concentration, bound is the measured binding at this concentration, and K_D the dissociation constant determined from saturation curves. The values for DA contents from Table 3 were converted to picomoles per milligram of protein (pmol/mg p.). The turnover index (TI) is the ratio between total metabolite content (DOPAC + HVA + 3-MT) and endogenous DA. (Modified from Dewar and Reader, 1989b; Dewar et al., 1989,1990; Diop et al., 1988; and Reader et al., 1988a).

postnatally in rat rostral and caudal neostriatum (D_1 and D_2 binding sites) as well as in the cerebral cortex (D_1 binding sites). In the rostral neostriatum, D_1 and D_2 receptor densities progressively increase, whereas endogenous DA augments from postnatal d 30–90 and there is a slight decrease in the turnover index (TI), determined as the ratio between DA metabolites and DA (Table 8). In the caudal neostriatum, measurements were conducted only at 30 and 90 d, but again show an increase in D_1 and D_2 receptor densities as well as in endogenous DA; however, the TI was found to be decreased. In the cerebral cortex, there was a gradual and progressive augmentation in D_1 receptor densities from 70 to 140 fmol/mg. Although DA content was unchanged,

Table 8
Postnatal Developmental Parameters of Rat Neostriatal and Cortical Dopamine Systems

	15 days	30 days	90 days	F
		Rostral neostriatum		
D_1 receptors				
B_{max}	287.00 ± 58[a]	763.00 ± 122[e]	1117.00 ± 137[d]	14.058
K_D	0.70 ± 0.15	0.42 ± 0.12	0.33 ± 0.04[b]	2.901
D_2 receptors				
B_{max}	188.00 ± 31[a]	293.00 ± 36	358.00 ± 11[c]	9.283
K_D	0.57 ± 0.02	0.73 ± 0.13	0.68 ± 0.05	1.015
D_1/D_2	1.53	2.60	3.12	—
Dopamine	357.00 ± 51	358.00 ± 39[f]	651.00 ± 57[d]	11.626
TI	2.09	1.11	0.87	—
		Caudal neostriatum		
D_1 receptors				
B_{max}	ND	380.00 ± 67	573.00 ± 73	3.662
K_D	ND	0.58 ± 0.13	0.45 ± 0.03	1.136
D_2 receptors				
B_{max}	ND	137.00 ± 21	180.00 ± 12	2.877
K_D	ND	0.92 ± 0.08	0.93 + 0.17	0.003
D_1/D_2	—	2.77	3.18	—
Dopamine	ND	199.00 ± 40	303.00 ± 81	1.608
TI	—	0.76	1.00	—
		Cerebral cortex		
D_1 receptors				
B_{max}	71.00 ± 11[a]	109.00 ± 8[e]	138.00 ± 8[c]	13.541
K_D	0.68 ± 0.09	0.61 ± 0.08	0.65 ± 0.07	0.188
Dopamine	1.97 ± 0.30	1.97 ± 0.18	2.01 ± 0.19	0.012
TI	6.7	4.4	5.74	—

The results are the means ±SEM (n = 6–18). The B_{max} values are in femtomoles per milligram of protein and the dissociation constants (K_D) in nanomolar (n*M*). The levels of DA are in picomoles per milligram of protein and the turnover index (TI) was calculated from the ratio between metabolites (DOPAC + HVA + 3-MT) and DA. The *F* values were obtained by a one-way analysis of variance (T&ANOVAR; Barlow, 1983), and the significant differences between the age groups had the following probabilities: [a]$p < 0.05$, between 15 and 30 d; [b]$p < 0.05$, between 15 and 90 d; [c]$p < 0.01$, between 15 and 90 d; [d]$p < 0.001$, between 15 and 90 d, [e]$p < 0.05$, between 30 and 90 d, and [f]$p < 0.001$, between 30 and 90 d. ND = not determined. (Modified from Dewar et al., 1990.)

suggesting that in this region, the dopaminergic innervation is established earlier than in the neostriatum and that the increase in cortical D_1 receptor number is seemingly independent of the dopaminergic input. In conclusion, in the three regions, there is a progressive increase in D_1 and D_2 receptor density that can be correlated in the neostriatum but not the cerebral cortex with endogenous DA. Since cortical DA may also be contained in noradrenergic terminals, a more meaningful interpretation of the developmental data calls upon the future use of markers of the DA uptake recognition site to quantify the number of DA nerve endings.

Summary

The biochemical properties of CNS dopamine D_1 and D_2 receptors were examined using the specific antagonists [^{3}H]SCH23390 and [^{3}H]raclopride, respectively. Treatment of membranes with L-DTT and NEM produced dose-dependent decreases in binding of both ligands. Saturation curves showed that after reduction of -SS- bonds by L-DTT, the B_{max} of D_1 and D_2 sites was decreased. Alkylation of -SH groups by NEM decreased [^{3}H]SCH23390 binding through an affinity change; in contrast, the affinity and B_{max} of [^{3}H]raclopride were affected. In competition experiments, neither L-DTT nor NEM significantly altered IC_{50} values for the D_1 agonist SKF38393, indicating that D_1 agonist sites remained as in control membranes. The antagonist affinity of D_2 sites were conserved, i.e., the IC_{50} values for the antagonist (+)butaclamol in control and in treated membranes were unchanged with Hill coefficients (n_H) of 1. Inhibition curves by DA of [^{3}H]raclopride binding were shallow ($n_H < 1$) and the IC_{50} greater in treated membranes than in controls.

The ionic regulation of D_1 and D_2 receptors also was examined with membrane preparations from neostriatum and cerebral cortex. Saturation binding of [^{3}H]SCH23390 in the presence of either Na^+ or Li^+ revealed an increase in the affinity, as compared to that observed in their absence. For the neostriatum, there were no changes in B_{max} in the different buffers but for the cortex; there was a loss of [^{3}H]SCH23390 sites when either Na^+ or Li^+ was added, suggesting a lack of selectivity of this ligand in the absence of group IA cations. The agonist state of the [^{3}H]SCH23390 site was studied in competition experiments with DA. The addition of either Na^+ or Li^+ caused a three- to fivefold decrease in the potency of DA to compete with [^{3}H]SCH23390 binding in both tissues.

In the case of neostriatal D_2 receptors, Na^+ and Li^+ produced dose-dependent elevations in specific [^{3}H]raclopride binding, with Na^+ inducing 50% more binding than Li^+. Inhibition of [^{3}H]raclopride binding by (+)butaclamol was unaffected by the presence of Na^+ or Li^+; in contrast, the potency of DA to compete with [^{3}H]raclopride was decreased by these two ions. This effect was more pronounced in the presence of Na^+ than Li^+ and was observed for both the high- and low-affinity states of the D_2 receptor. Distribution of D_1 and D_2 binding sites was measured in rat and rabbit cortical areas, hippocampus, and neostriatum. Dopamine and its metabolites DOPAC, HVA, and 3-MT were assayed in these same regions by HPLC. The distribution of D_1 and D_2 receptors was heterogenous in both species, with the greatest densities in the neostriatum, where the highest concentrations of DA and its metabolites were measured. Regions with low endogenous DA content (cerebral cortex and hippocampus) had lower densities of DA receptors. Furthermore, these binding sites were differentially localized within the various regions, and there were substantially more D_1 than D_2 receptors. Finally, in rat cerebral cortex, rostral, and caudal neostriatum, there was a progressive increase from postnatal d 15–90 in DA receptor densities that could be correlated with endogenous DA.

The studies reviewed here confirm the existence of a DA system throughout the cerebral cortex of rat and rabbit, including the presence of two pharmacologically-distinct DA receptors with different ionic regulatory properties and participation of -SS- and -SH. The biochemical assays demonstrate very low trace levels (Reader et al., 1988b) of endogenous DA in neocortical areas; however, the coexistence of metabolites warrant that DA is released from nerve terminals. The responses to the iontophoretic application of DA acting via D_1 receptors in visual cortex (Gottberg, Montreuil, and Reader, 1988) also favor a dopaminergic innervation in this neocortical region. Although DA D_1 receptors throughout the CNS are seemingly more abundant than D_2 sites, the functional roles and interactions of both receptor subtypes in higher mental functions deserve further elucidation.

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