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Distinctions between Ligand-Binding Sites for [^{3}H]Dopamine and D_2 Dopaminergic Receptors Characterized with [^{3}H]Spiroperidol

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SUMMARY

The binding of [^{3}]Hspiroperidol to D_2 dopaminergic receptors in rat striatum was compared to the binding of [^{3}H]dopamine to its binding sites. Both radioligands labeled apparently homogeneous populations of high affinity, stereoselective, saturable sites, determined from analysis of saturation isotherms. [^{3}H]Spiroperidol bound to more than twice as many sites as [^{3}H]dopamine, and antagonist/[^{3}H]spiroperidol competition data were consistent with a single population of receptors. Antipsychotic drugs competed with both high and low affinities for two fractions of [^{3}H]dopamine-binding sites, but for most drugs, their potencies at even the high affinity component were significantly less than their affinities at D_2 receptors. The [^{3}H]dopamine-binding sites were altered by kainic acid lesions of the striatum, phenoxybenzamine treatment of tissue homogenates, or reserpine pretreatment of rats. These changes were different from previous reports of alterations in either D_2 or D_1 dopaminergic receptors. These and other differences in binding properties suggest that [^{3}H]dopamie binds to sites distinct from either D_1 or D_2 receptors.

INTRODUCTION

Over the past several years, dopamine receptor systems have been studied extensively but not classified uniformly. Kebabian and Calne (1) divided dopamine receptors into a D_1 type, linked to the stimulation of adenylate cyclase by dopamine, and a D_2 type, apparently not linked to activation of adenylate cyclase. Most investigators agree with this classification but others feel it does not adequately describe their data and have introduced the concepts of D_3 or even D_4 receptors (for reviews, see refs. 2 and 3). Considerable controversy over receptor characteristics and nomenclature has resulted (3, 4).

The focus of much of this debate centers on radioligand binding to dopaminergic receptors in the brain. Investigators currently have at their disposal a plethora of labeled ligands for dopamine receptors. This diversity of ligands and the variety of experimental conditions used to optimize data acquisition have contributed to the confusion about the types of dopamine receptors and their relevance to disease processes.

Currently, only D_1 and D_2 receptors can be linked to the pharmacological and physiological effects of dopaminergic agonists and antagonists. Although the D_1 receptor can be defined by pharmacological means using dopamine stimulation of adenylate cyclase, no pathophysiological condition has yet been correlated to activation or blockade of D_1 receptors. The D_2 receptor is generally recognized as the site of action of antipsychotic drugs. There is an excellent correlation between drug potency at these receptors in striatal tissue (judged from radioligand binding) and both behavioral actions of antipsychotic drugs in animal models predictive of antipsychotic action and therapeutic doses of neuroleptic drugs in schizophrenic patients (5). Neuroleptic drugs exhibit high affinity for these receptors. Radiolabeled butyrophenones, most recently [^{3}H]spiroperidol, have been used extensively to study these D_2 receptors. In contrast, the striatal D_3-binding site cannot be considered a receptor since no pharmacological effect has yet been associated with binding to this site (3). Binding studies typically use tritium-labeled agonists (dopamine, apomorphine) to characterize D_3 sites.

One basic way to compare D_2 receptors and D_3 ligand-binding sites has been to use potency relationships among dopaminergic agonists or antagonists. Antagonists are generally of higher potency at D_2 receptors than at D_3 sites whereas the opposite is true for agonists (6). In the past, rank orders of potency have been obtained based on the IC_{50} values (concentration of drugs required to inhibit by 50% the specific binding of the radioligand) or the K_I values [inhibition constants derived from IC_{50} by the correction of Cheng and Prusoff (7)]. However, agonist binding to D_2 receptors and antagonist binding to D_3 sites suggest heterogeneous receptors because the displacement curves are shallow [Hill coefficients (nH) of less than 1]. Thus, as Creese and Sibley (4) have indicated, the IC_{50} of such a competition curve is an average of inhibitory actions at receptors or sites of different affinities. Therefore, calculation of the K_I is not

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only inappropriate, but has the shortcoming of failing to describe and characterize fully the nature of the drug-receptor interactions.

A more appropriate approach to analyze heterogeneous receptor-binding data is the use of nonlinear least squares analysis. This method has been used to study mixtures of receptor subtypes (8) and different affinity states of a single receptor (9). The method is a direct extension of mass action law and has been thoroughly tested under both theoretical and experimental conditions (10). The advantage of this technique is that the individual K_D values (dissociation constants of radioligands or competitor drugs) and receptor numbers can be directly ascertained from untransformed data for each of several components of a heterogeneous receptor mixture.

We have used this mathematical approach to characterize more fully the striatal receptors or sites to which dopaminergic agonists and antagonists bind based on radioligand-binding data. Our results indicate that there are substantial differences between D_2 receptors labeled with [^{3}H]spiroperidol and the binding sites labeled with [^{3}H]dopamine (previously called D_3 sites). A preliminary report of some of these data has been published (11).

MATERIALS AND METHODS

Striatal tissue (12) was obtained from male Wistar rats (Harlan Industries, Inc., Indianapolis, IN) at approximately 2 p.m. (13) and frozen immediately on dry ice. When tissues were stored at −70°, the binding parameters were stable for months.

In some experiments, rats anesthetized with sodium pentobarbital (60 mg/kg intraperitoneally) received unilateral intrastriatal injections of kainic acid. The method was as reported (14) with the exception that the contralateral striatum was injected with vehicle. Kainic acid (2 μg/1 μl in 0.9% saline) was injected into the striatum [8.9 mm anterior, 2.7 mm lateral, and −5.4 mm vertical (15)]. Three weeks later, lesioned rats were treated with 3.0 mg/kg apomorphine intraperitoneally in 3 mg/ml ascorbic acid and examined for ipsilateral rotational behavior for the next 20 min, at which time peak-turning effects are observed (16). Those rats which failed to turn ipsilaterally more than 40 rotations were not used. Brains from randomly selected rats that passed the turning test were examined histologically, and the lesions were as previously described (17). The remaining rats were killed 1 week after the rotational tests and their striata were removed and frozen, as above. In addition, another group of rats was treated 21 hr prior to sacrifice with reserpine [4.0 mg/kg subcutaneously (18)].

D_2 receptor binding was performed essentially as described (19) with minor modifications. Striatal tissue was homogenized in ice-cold 50 mM Tris (pH 7.7) using a Polytron, the homogenate was centrifuged at 50,000 × *g* for 10 min, and the pellet was resuspended in 50 mM Tris/0.1% ascorbic acid (pH 7.1). The washed homogenate was recentrifuged, resuspended as above, and centrifuged a final time. The final pellet was resuspended in 200 volumes (per g original wet weight) Tris/ascorbate and used in the assay. Each glass assay tube (2-ml final volume) included 1.3 ml of Tris/ions mixture (to yield a final concentration of 50 mM Tris, 60 mM NaCl, 2.5 mM KCl, 1.0 mM $CaCl_2$, 0.50 mM $MgCl_2$, and 0.10 mM Na_2EDTA, pH 7.1), 200 μl of Tris/ascorbate or unlabeled competitor drug in Tris/ascorbate, 100 μl of [^{3}H]spiroperidol (22–33 Ci/mmol; New England Nuclear, Boston, MA) in 0.1% ascorbic acid, and 400 μl of the membrane preparation (2 mg wet weight, approximately 300 μg of protein) and incubated for 15 min at 37°. Nonspecific binding was defined using 1 μM (+) butaclamol. Bound ligand was separated from free by rapid vacuum filtration over GF/B filters with 4 × 5 ml washes of the filters with 50 mM Tris buffer. Membrane protein was digested for 30 min with 500 μl of Protosol (New England Nuclear) and a toluene-based scintillation fluid (Omnifluor, New England Nuclear, containing 0.5% glacial acetic acid to reduce chemiluminescence) was added. Following 6 or more hr of equilibration, vials were counted by liquid scintillation spectroscopy and corrected for quench, and the data were converted to disintegrations per min. Protein content of homogenates was determined using the method of Lowry *et al.* (20).

[^{3}H]Dopamine binding (41.5 Ci/mmol, New England Nuclear) was performed essentially as previously described (21), with experiments including phenoxybenzamine modified from a previous report (6). Briefly, striata were homogenized using a Polytron in 100 volumes of cold 50 mM Tris (pH 7.7), centrifuged as above, and resuspended in 50 volumes of 50 mM Tris/2 mM $MgSO_4$, pH 7.7. Membranes were incubated for 5 min at 37°. If appropriate, sufficient phenoxybenzamine was added in 100 μl of 10% ethanol to yield a 10 μM concentration in the homogenate. Controls contained 100 μl of vehicle. The homogenates were incubated a further 10 min at 37°, then diluted with 50 volumes of 50 mM Tris, and centrifuged. The pellets were washed twice with 100 volumes of 50 mM Tris with intermediate centrifugation, and finally resuspended in 160 volumes of buffer consisting of 25 mM 4-morpholinepropanesulfonic acid, 1.25 mM EDTA, 1.25 mM ascorbic acid, 4 mM $MgSO_4$, and Tris base to pH 7.2. In the assay, each tube (1-ml final volume) included 100 μl of Tris/ascorbate buffer with or without cold competitor drug, 100 μl of [^{3}H]dopamine in 0.1% ascorbic acid, and 800 μl of membrane preparation (5 mg wet weight, approximately 750 μg of protein). Nonspecific binding was defined using 2 μM *cis-z*-flupentixol. Reagents were incubated 60 min at 22° followed by vacuum filtration and scintillation counting as described above. For comparative purposes, the same membrane isolation procedures and assay techniques were also used for certain [^{3}H]spiroperidol binding experiments.

As indicated above, different competing ligands were used to define the nonspecific binding of [^{3}H]spiroperidol [1 μM (+) butaclamol] and [^{3}H]dopamine (2 μM *cis-z*-flupentixol). These compounds were chosen as they are those commonly employed in the literature for defining the nonspecific binding of these radioligands. However, the choice of the competing ligand actually turns out to be immaterial, for two reasons. First, as shown later in Figs. 1 and 2, various competing drugs all approach the same level of nonspecific binding. Second, and more important, in the mathematical analysis of the data described below, nonspecific binding is not a part of the analytical data set itself when analyzing competition binding using the SCAFIT or ALLFIT computer programs. Rather, nonspecific binding becomes an estimated parameter, based upon the minimal amount of binding of several competitors assayed simultaneously.

Data were analyzed in one of several ways. For saturation isotherms, specific binding was converted into Scatchard (22) plots and analyzed using least squares techniques. In addition, untransformed total or specific binding data were analyzed using a nonlinear least squares program [SCAFIT (8, 23)] to test for radioligand binding to heterogeneous receptor populations.

Data from competition assays also were not transformed prior to analysis. Groups of concentration-response curves were first analyzed using a four-parameter logistic equation [ALLFIT (8, 24)]. From this analysis IC_{50} values and pseudo-Hill coefficients were obtained. If the latter were significantly less than 1, the hypothesis of different receptor subtypes of affinity states was suggested. All competition assay data were further analyzed using the nonlinear least squares SCAFIT program as described above, testing for evidence of binding to one or more receptor subtypes. From this analysis, we obtained estimates of K_D values of each drug at those receptors or sites to which it bound and the concentration of those receptors (which could be directly converted to femtomoles of receptor per mg of protein). The geometric means of K_D values from several experiments using each drug were calculated (10). The values obtained in competition assays for [^{3}H]spiroperidol or [^{3}H]dopamine were compared using Student's *t* test.

Drugs used in this study were obtained from the following sources: McNeil Laboratories, Inc., spiroperidol, haloperidol, droperidol, triflu-

peridol hydrochloride, and pipamperone dihydrochloride; Smith, Kline and French Laboratories, chlorpromazine hydrochloride, trifluoperazine hydrochloride, and phenoxybenzamine; Sandoz Pharmaceuticals, thioridazine and clozapine; H. Lundbeck and Co., piflutixol and *cis*-*z*-flupentixol dihydrochloride; Ayerst Research Laboratories, (+) and (−)butaclamol hydrochloride; Sigma Chemical Company, dopamine hydrochloride, bromocriptine methane sulfonate, kainic acid, and 5′-guanylylimidodiphosphate; Merck Chemical Division, apomorphine hydrochloride; Merck, Sharpe and Dohme Research Laboratories, metoclopramide hydrochloride; Lederle Laboratories, loxapine succinate; Endo Laboratories, molindone hydrochloride; Labs Dela Grange, sulpiride; S. B. Penick, reserpine. Lenperone hydrochloride and 3-PPP[1] were synthesized in our own laboratories.

RESULTS

The binding of [^{3}H]spiroperidol to rat striatal D_2 receptors and the binding of [^{3}H]dopamine to its binding sites were each of high affinity, saturable, stereoselective, consistent with binding to a homogeneous population of receptors for each ligand, and possessing a high ratio of specific to total binding (80–90% at the K_D of each ligand). Table 1 provides a synopsis of the relevant parameters obtained by least squares regression analysis of Scatchard or Hill plots (not shown). The usual concentrations of [^{3}H]spiroperidol used for saturation isotherms were 0.01 to 0.40 nM. Computerized nonlinear least square analysis using the SCAFIT program did not detect any binding of [^{3}H]spiroperidol to more than one site even when the range of ligand was extended to 8 nM and 25 concentrations of radioligand were employed. For [^{3}H]dopamine, usual ligand concentrations ranged from 0.1 to 10 nM, and analysis was always consistent with a single homogeneous population of binding sites.

Agonist competition for [^{3}H]spiroperidol binding. We studied the binding of agonists to D_2 receptors in competition assays between [^{3}H]spiroperidol and dopaminergic agonists, bromocriptine, dopamine, apomorphine, and 3-PPP (Tables 2 and 3). Bromocriptine is a unique dopaminergic agonist. Previous reports have suggested irreversible binding to dopamine receptors using *in vivo* techniques (25) although this was not confirmed in radioligand-binding assays (26). In our experiments, bromocriptine acted as an irreversible ligand at D_2 receptors. Preincubation of striatal membranes for 60 min with 1 nM bromocriptine caused an apparent 3-fold increase in the K_D of [^{3}H]spiroperidol and a 43% decrease in the B_{max} (maximum density of receptors) following a 30-min incubation with the radioligand. These changes were consistent with the irreversible occupancy of the receptors by bromocriptine. In the absence of the preincubation, only the K_D was altered. Also, by increasing the length of time from 15 to 60 min that the membranes were incubated concomitantly with [^{3}H]spiroperidol and

TABLE 1

Comparison of binding of [^{3}H]spiroperidol to binding of [^{3}H]dopamine in rat striatal membranes

K_D and B_{max} values and Hill coefficients (nH) were derived from least squares regression analysis of Scatchard or Hill plots, respectively. Saturation assays were performed as described under Materials and Methods. The range of concentrations of radioligand was generally 0.01 to 0.40 nM for [^{3}H]spiroperidol and 0.1 to 10 nM for [^{3}H]dopamine binding. Values for K_D are geometric means (with 95% confidence limits) and for B_{max} are the mean ± standard error of the mean of n experiments.

Ligand	K_D	B_{max}	nH	n
	nM	*fmol/mg protein*		
[^{3}H]Spiroperidol	0.052 (0.049–0.055)	184 ± 6	1	66
[^{3}H]Dopamine	1.4 (1.2–1.7)	76 ± 9	1	7

[1] The abbreviations used are: 3-PPP, 3-(1-propyl)piperidinyl phenol; Gpp(NH)p, 5′-guanylylimidodiphosphate; 6-OH-DA, 6-hydroxydopamine.

TABLE 2

Quantitative analysis of agonist affinities in the presence and absence of Gpp(NH)p as calculated from competition assays for [^{3}H]spiroperidol binding to rat striatal membranes

K_H and K_L represent dissociation constants for the agonist high and low affinity conformations of the D_2 receptor, respectively, observed in the absence of added guanine nucleotide. Gpp(NH)p shifted the competition curves to the right and steepened them, giving results consistent with a single population of lower affinity receptors as shown by the similar dissociation constants K_L [in the absence of Gpp(NH)p] and K_D [in the presence of Gpp(NH)p]. All values are geometric means (with 95% confidence limits) derived from computer analysis using the nonlinear least squares curve-fitting program SCAFIT. The number of experiments is given by n.

Agonist	Control			Gpp(NH)p (0.1 mM)	
	K_H	K_L	n	K_D	n
	nM			*nM*	
Apomorphine	1.7 (1.5–1.9)	64 (52–78)	4	50 (29–85)	3
Dopamine	16 (10–25)	1100 (750–1600)	8	800 (430–1500)	3
3-PPP	36 (21–63)	1100 (810–1500)	4	880 (760–1000)	3
Bromocriptine	3.9 (3.2–4.8)		3	1.0 (0.49–2.0)	3

TABLE 3

Receptor concentrations in high or low agonist affinity conformations and their modification by Gpp(NH)p

Data were derived from agonist competition assays against [^{3}H]spiroperidol binding as described under Materials and Methods. R_H and R_L represent the concentrations of agonist high or low affinity conformation of the binding sites, respectively. R_T is the summation of R_H and R_L for a given agonist and the group average is R_T. R_G represents the concentration of receptors in the presence of Gpp(NH)p, and the group average is given by R_G. Values are the mean ± the standard error of the mean for n experiments.

Agonist	Control				Gpp(NH)p (0.1 mM)	
	R_H	R_L	R_T	n	R_G	n
	fmol/mg protein				*fmol/mg protein*	
Apomorphine	119 ± 23	96 ± 14	249 ± 43	4	233 ± 80	3
Dopamine	112 ± 14	106 ± 17	219 ± 19	8	187 ± 27	3
3-PPP	57 ± 5	122 ± 39	176 ± 39	4	185 ± 53	3
Bromocriptine	170 ± 2	0	170 ± 2	3	144 ± 12	3
R_T			212 ± 16	19		
R_G					187 ± 23	12

bromocriptine, we observed a 2-fold increase in the apparent affinity of bromocriptine for D_2 receptors. These results differed from those observed with competitive, reversible ligands (27). Bromocriptine competition curves were steep ($nH = 1$) and were not altered by the addition of Gpp(NH)p, as reported previously (28).

The other agonists we studied, dopamine, apomorphine, and 3-PPP, had shallow competition curves ($nH < 1$), an observation consistent with more than one agonist affinity state of the D_2 receptor and in agreement with previous reports (19, 28, 29). The effects of guanine nucleotides (see below and also refs. 19, 28, 29) supported the hypothesis of different agonist affinity states for D_2 receptors as opposed to different receptor subtypes. For both dopamine and apomorphine, approximately one-half of the receptors for which they competed were of high agonist affinity, whereas only one-third of the receptors for which 3-PPP competed were of high affinity. This latter observation would be consistent with reports of different actions of 3-PPP enantiomers (30). Thus, (−)3-PPP acts as a postsynaptic antagonist and, therefore, should bind to a homogeneous population of sites (one-half of the total). In contrast, (+)3-PPP is an agonist and would be expected to bind with both high and low affinity to [^{3}H]spiroperidol-binding sites. Therefore, of the total number of binding sites, less than 50% might be expected to be in the high affinity conformation when occupied by (+) or (−)3-PPP.

As shown in Table 2, all three reversible agonists were between 38- and 69-fold selective for the high affinity conformation for the D_2 receptors. The addition of Gpp(NH)p to these agonist competition assays produced curves which were shifted to the right and which generally had a single K_D similar to that of the low affinity K_D of the same agonist in the absence of Gpp(NH)p (Table 2). In addition, the concentration of receptors in the presence of Gpp(NH)p was the same as the total concentration of receptors in both high and low affinity conformations in the absence of Gpp(NH)p (Table 3). Thus, the reversible agonists apomorphine, dopamine, and 3-PPP appeared to bind to heterogeneous states of the D_2 receptor which could be shifted to a homogeneous, low affinity state by the addition of the guanine nucleotide Gpp(NH)p. The binding of bromocriptine, an irreversible agonist, appeared to be to a homogeneous population of D_2 receptors and was unaffected by the addition of Gpp(NH)p.

Antagonist competition for [^{3}H]spiroperidol binding. In contrast to the complex agonist competition at D_2 receptors, antagonist competition was simpler, consistent with competitive, reversible binding to a homogeneous population of sites. Figure 1 illustrates competition assay curves for several antagonists of different chemical classes. The curves were all steep ($nH = 1$), uniphasic, and consistent with the reaction sequence of drug plus receptor yielding a bimolecular drug-receptor complex ($D + R \rightleftharpoons DR$). These conclusions were based on analysis using both ALLFIT and SCAFIT. Using these programs, data were successively fit to models of one or two sites. Generally, the data failed to support a two-site model, manifested either as a lack of convergence to a two-site fit, or more frequently, as a setting of the concentration of the second site to zero. In those cases where a two-site model could be derived from the data, the analysis showed no significant improvement of the fit to the two-site model. Therefore, the data supported a simple one-receptor model as summarized in Table 4. Butyrophenones were of high affinity for D_2 receptors, as expected. The K_D of nonradioactive spiroperidol agreed closely to that of [^{3}H]spiroperidol obtained using saturation binding conditions. The binding of D_2 receptors was also highly stereoselective (2800-fold for the isomers of butaclamol). In addition, we calculated the total number of receptors labeled, based on SCAFIT analysis of both agonist and antagonist competition curves, incorporating the results for agonists listed in Table 3 plus additional data for antagonists. This value was 188 ± 8 fmol/mg of protein ($n = 43$) and did not differ significantly from 184

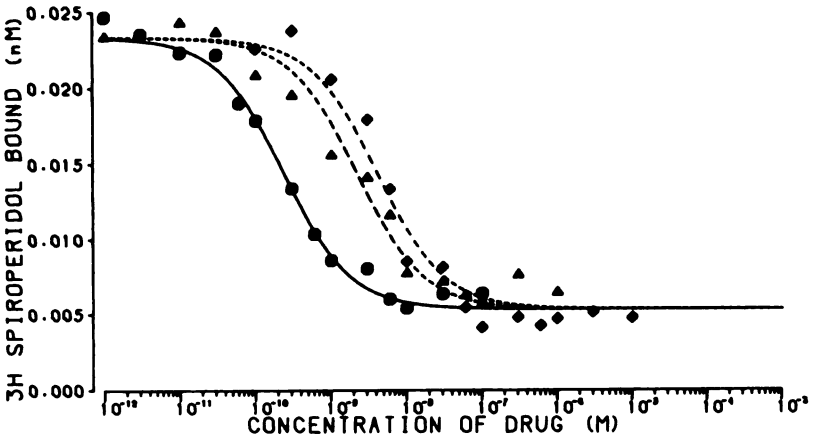

FIG. 1. *Competition between [^{3}H]spiroperidol and dopaminergic antagonist drugs*

The figure illustrates a typical experiment using the antagonists spiroperidol (●), piflutixol (▲), or trifluoperazine (◆) at D_2 receptors in rat striatum labeled with [^{3}H]spiroperidol. The total receptor concentration was 173 fmol/mg of protein. The K_D values of competitor drugs were 0.046 nM for spiroperidol, 0.42 nM for piflutixol, and 0.78 nM for trifluoperazine. These parameters were derived from computer analysis of the assay results using SCAFIT. The symbols represent actual data points obtained, whereas the lines are computer generated from the best-fit parameters. The binding assay followed simple mass action kinetics.

TABLE 4

Dissociation constants of antagonists for D_2 receptors labeled with [^{3}H]spiroperidol grouped according to chemical class

K_D represents the dissociation constants derived from analysis of competition assays using SCAFIT. Values are the geometric means (and 95% confidence limits) of n experiments.

Antagonist	K_D		n
	nM		
Butyrophenones			
Droperidol	0.81	(0.77–0.86)	3
Haloperidol	0.64	(0.54–0.77)	6
Lenperone	5.9	(5.0–6.9)	3
Pipamperone	36	(32–40)	3
Spiroperidol	0.061	(0.051–0.073)	4
Trifluperidol	0.48	(0.43–0.53)	3
Phenothiazines			
Chlorpromazine	1.7	(1.4–2.0)	3
Thioridazine	3.3	(2.8–3.9)	4
Trifluoperazine	1.2	(1.1–1.3)	3
Benzamides			
Metoclopramide	99	(95–103)	3
Sulpiride	85	(76–95)	3
Thioxanthines			
cis-z-Flupentixol	0.72	(0.69–0.76)	3
Piflutixol	0.54	(0.41–0.69)	3
Other			
(+)Butaclamol	0.52	(0.49–0.55)	3
(−)Butaclamol	1400	(810–2500)	3
Clozapine	50	(40–62)	3
Loxapine	4.3	(3.4–5.6)	3
Molindone	27	(23–31)	3

± 6 fmol/mg of protein derived from Scatchard analysis of 66 saturation isotherms (Table 1).

Agonist and antagonist competition for [^{3}H]dopamine binding. The patterns of agonist and antagonist competition at sites labeled with [^{3}H]dopamine were almost the inverse of those described above for [^{3}H]spiroperidol binding. Most antagonist competition curves were shallow ($nH < 1$), complex, and revealed a heterogeneity of [^{3}H]dopamine-binding sites (Figs. 2–5). Most agonist competition curves were steep ($nH = 1$, Tables 1 and 5), uniphasic, and consistent with a bimolecular reaction of drug plus receptor at a homogeneous population of binding sites. For agonists, the maximal number of sites derived from computer analysis of competition curves was approximately 60 fmol/mg of protein (Table 5), comparable to the value ascertained from saturation experiments with [^{3}H]dopamine (76 fmol/mg of protein, Table 1). In addition, the K_D of nonradioactive dopamine was similar to the value obtained for [^{3}H]dopamine in direct binding experiments (*cf.* Tables 1 and 5). Because guanine nucleotides substantially decrease the affinity of agonists at [^{3}H]dopamine sites (21), no experiments analogous to those described in Table 2 were performed for agonist/[^{3}H]dopamine competition.

Bromocriptine and 3-PPP both differed from the other agonists we studied in their binding to [^{3}H]dopamine sites. Bromocriptine appeared to bind irreversibly to these sites using conditions analogous to those described above at D_2 receptors. Experiments in which the striatal homogenates were preincubated for 60 min demonstrated that 25 nM bromocriptine irreversibly occupied approximately 30% of the [^{3}H]dopamine-binding sites. All of these sites were irreversibly occupied if sufficient bromocriptine were added first. In experiments using 3-PPP, this compound displayed heterogeneous binding to [^{3}H]dopamine sites, with approximately 50% of the receptors having high affinity for 3-PPP. This result may be due to differences in the affinities of the (+) and (−) enantiomers of 3-PPP for these sites (30).

Excepting 3-PPP, each agonist had a single affinity for all [^{3}H]dopamine-binding sites. In contrast, antagonist drugs had both high and low affinities for [^{3}H]dopamine-binding sites as shown in competition experiments. Fig. 2 illustrates some typical competition curves of antagonists at [^{3}H]dopamine-binding sites. Butyrophenones were of very high affinity for approximately

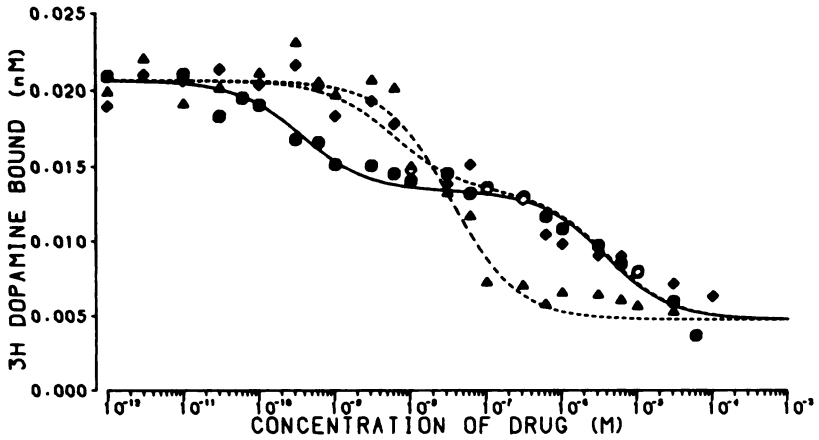

FIG. 2. *Competition between [^{3}H]dopamine and dopaminergic antagonist drugs*

The figure illustrates a typical experiment using the antagonists spiroperidol (●), piflutixol (▲), or trifluoperazine (◆) at binding sites in rat striatum labeled with [^{3}H]dopamine. The concentration of binding sites in the antagonist high affinity component (R_H) was 35 fmol/mg of protein, or 46% of the total. The concentration of binding sites in the antagonist low affinity component (R_L) was 42 fmols/mg of protein, or 54% of the total (77 fmol/mg of protein). The high and low affinity dissociation constants of the competitor drugs were K_H = 0.23 nM and K_L = 2200 nM for spiroperidol, K_H = 3.7 nM and K_L = 2400 nM for trifluoperazine. For piflutixol, binding followed simple mass action kinetics and $K_H = K_L$ = 21 nM. Parameters were derived from computer analysis using SCAFIT. Symbols and lines are as described in Fig.1.

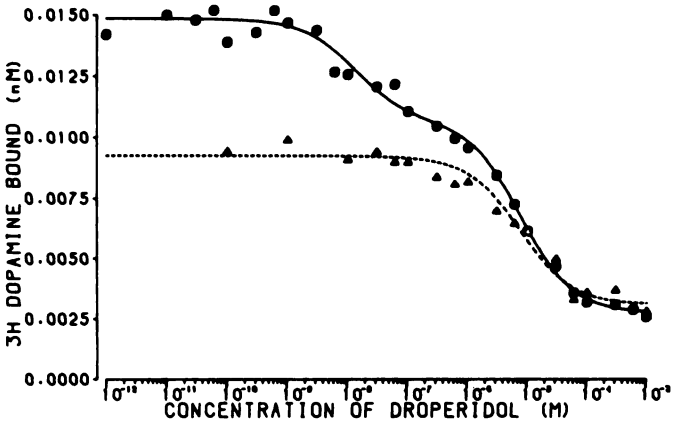

FIG. 3. *Effect of phenoxybenzamine on [³H]dopamine-binding sites*

The figure illustrates a typical competition experiment using the antagonist droperidol at binding sites in the rat striatum labeled with [³H]dopamine. Membranes were incubated with either vehicle (●) or phenoxybenzamine (10 μM, ▲) for 10 min as described under Materials and Methods. The parameters calculated from this data using SCAFIT for the control experiment were: K_H = 8.0 nM, K_L = 4700 nM, R_H = 16 fmol/mg of protein, or 34% of the total and R_L = 30 fmol/mg of protein or 66% of the total (46 fmol/mg of protein). Phenoxybenzamine caused the elimination of binding to those sites for which droperidol had high affinity. The parameters describing the remaining binding sites were K_L' = 4600 nM and R_L' = 24 fmol/mg of protein. The number of low affinity sites was not significantly reduced by phenoxybenzamine ($R_L = R_L'$). Symbols and lines are as described in Fig. 1.

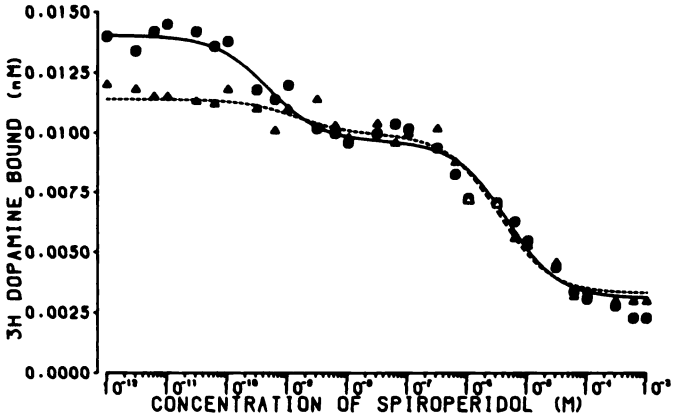

FIG. 4. *Effect of intrastriatal kainic acid on [³H]dopamine-binding sites*

The figure illustrates a typical competition experiment using the antagonist spiroperidol at binding sites in the rat striatum labeled with [³H]dopamine. Saline (●) or kainic acid (▲) were injected intrastriatally as described under Materials and Methods. The parameters calculated from this data using SCAFIT for the control experiment were K_H = 0.32 nM, K_L = 3000 nM, R_H = 15 fmol/mg of protein or 40% of the total, R_L = 22 fmol/mg of protein or 60% of the total number of [³H] dopamine-binding sites (37 fmol/mg of protein). For the striata from rats lesioned with kainic acid, the parameters obtained from this experiment were K_H' = 0.92 nM, K_L' = 2200 nM, R_H' = 5 fmol/mg of protein or 18% of the total, and R_L' = 23 fmol/mg of protein or 82% of the total (28 fmol/mg of protein). In this assay, R_H' (kainic acid lesioned) was 67% less than R_H (control). The antagonist high and low affinity dissociation constants were not affected by the kainic acid lesions compared to control. Similarly, the number of sites in the antagonist low affinity conformation (R_L') was not reduced by the lesion. Symbols and lines are as described in Fig. 1.

50% of the [³H]dopamine-binding sites (Table 6), with K_D values in the nanomolar range, whereas the remainder of the sites possessed micromolar affinities, similar to previous reports (6, 21). Other antagonists also discriminated between high and low affinity [³H]dopamine-binding sites, and this selectivity extended over all classes of antipsychotic drugs tested (Table 6). One drug, piflutixol, was an exception (Fig. 2). Competition curves of this drug at [³H]dopamine-binding sites were steep (nH = 1) and uniphasic. Therefore, only a single K_D could be determined (Table 6).

Since antagonists differentiated between two subtypes of [³H]dopamine-binding sites, we investigated the regulation of these sites by various pharmacological interventions. These changes in protocol included preincubation of striatal homogenates with phenoxybenzamine, lesioning of rat striata with kainic acid, or pretreatment of rats with reserpine.

Effects of phenoxybenzamine. Phenoxybenzamine (10 μM) selectively alkylates the high affinity component of butyrophenone binding to [³H]dopamine sites (6). We observed a similar selective loss of the high affinity component as shown in Fig. 3. The loss of the high affinity component of antagonist binding was observed for all compounds tested, in every chemical class of antipsychotic drug. After phenoxybenzamine preincubation, the remaining receptors were in a homogeneous population (nH = 1), and the number of remaining receptors and their affinity for antagonists were identical to the low affinity antagonist sites in the control experiments (Table 7, Fig. 3).

These experiments using phenoxybenzamine helped to clarify the characteristics of the antagonist high and low affinity components of [³H]dopamine-binding sites. Computer analysis of assays involving a 50:50 mixture of two binding sites of different affinity can provide equivocal results. It is not possible for the analysis to distinguish between the case of two drugs each having high affinity for the same site or the case where one of the drugs has high affinity while the other drug has low affinity for one of the two different sites. Only when the receptor mixture differs from a 50:50 mixture, or the receptor proportions are perturbed (e.g., phenoxybenzamine pretreatment) does it become clear for which of the two sites drugs are competing with higher affinity. We observed that all of the drugs tested had a higher affinity for the same (phenoxybenzamine-sensitive) fraction of sites.

Effects of kainic acid. Intrastriatal kainic acid injection has been reported to eliminate differing proportions of D_1, D_2, and D_3 receptors or sites, presumably by selectively destroying intrinsic neurons in the striatum and, hence, the postsynaptic receptors thereon. Seeman (2) reported that intrastriatal lesioning decreased the B_{max} of D_2 receptors approximately 50%. We have obtained similar data in our laboratory,[2] suggesting that approximately half of striatal D_2 receptors may be located postsynaptically. In contrast, D_1 receptors labeled with [³H] flupentixol appear to be exclusively postsynaptic since they can be completely eliminated by kainic acid lesions

[2] A. A. Hancock and C. L. Marsh, unpublished.

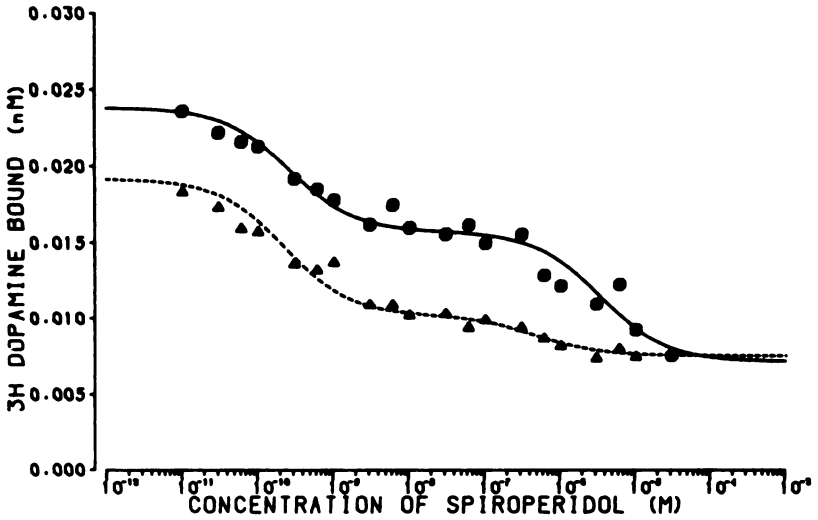

FIG. 5. *Effect of reserpine treatment of rats on [^{3}H]dopamine-binding sites*

The figure illustrates a typical competition experiment using the antagonist spiroperidol at binding sites in the rat striatum labeled with [^{3}H] dopamine. Saline (●) or reserpine (▲) was injected intraperitoneally 21 hr prior to removing the striata as described under Materials and Methods. The parameters calculated from this data using SCAFIT for the control experiment were K_H = 0.13 nM, K_L = 1800 nM, R_H = 26 fmol/mg of protein (49% of the total), and R_L = 28 fmol/mg of protein (51% of the total, 54 fmol/mg of protein). In reserpinized rats, K_H' = 0.11 nM, K_L' = 230 nM, R_H' = 26 fmol/mg of protein (77% of the total), and R_L = 7.6 fmol/mg of protein (23% of the total, 34 fmol/mg of protein). Reserpine significantly reduced the number of antagonist low affinity binding sites ($R_L' < R_L$, $p < 0.05$), thereby reducing total [^{3}H]dopamine binding. Symbols and lines are as described in Fig. 1.

TABLE 5

Characterization of agonist competition for [^{3}H]dopamine-binding sites

Competition assays were performed as described under Materials and Methods. K_H and K_L represent the dissociation constants (geometric means and 95% confidence limits) of drugs for the agonist high and low affinity conformations of the rat striatal [^{3}H]dopamine-binding sites. R_H and R_L represent the concentrations of high and low affinity binding sites for [^{3}H]dopamine, respectively. R_T is the total concentration of sites derived from all agonist competition data. Values given are the mean ± standard error of the mean of n experiments.

Agonist	K_H	K_L	R_H	R_L	n
	nM	*nM*	*fmol/mg protein*	*fmol/mg protein*	
Apomorphine	0.73 (0.58–0.93)		52 ± 4.6		3
Dopamine	3.0 (2.6–3.5)		60 ± 17		3
3-PPP	35 (25–50)	660 (470–910)	31 ± 5.9	33 ± 10	4
Bromocriptine	101 (84–121)		42 ± 4.5		3
R_T			59 ± 6		

(31). With respect to the D_3 site, more controversy exists. Kainic acid has been reported to decrease [^{3}H]*N*-propylnorapomorphine binding to the same extent as [^{3}H] flupentixol sites, suggesting the former ligand is binding to D_1 receptors (32). Binding of [^{3}H]apomorphine to D_3 sites has been reported to decrease 64% (33). However, D_3 binding of the same ligand was unchanged after kainic acid lesioning under different assay conditions (34). Using [^{3}H]dopamine to label D_3 sites, a 65% decrease in striatal ligand binding after kainic acid lesions was reported (35). In all of these studies, only B_{max} values of saturation assays were reported, leaving unanswered the question of which component of [^{3}H]agonist binding was most affected by kainic acid.

In our experiments, intrastriatal kainic acid reduced the B_{max} of [^{3}H]dopamine binding about 50% without changing the K_D of [^{3}H]dopamine for the remaining sites, results similar to those cited above. However, the binding to the antagonist high affinity sites of [^{3}H]dopamine was almost entirely eliminated by the lesion (Fig. 4), whereas [^{3}H]spiroperidol binding to D_2 receptors was reduced by only about 50%. The antagonist low affinity component of [^{3}H]dopamine binding was apparently not affected by kainic acid lesions, either in terms of numbers of binding sites or their affinity for competitor antagonists. These results suggested that only the antagonist high affinity sites have a postsynaptic location that would be destroyed by kainic acid lesions.

Our experiments also provided an important test with respect to the pharmacology of 3-PPP, a compound previously thought to be selective for presynaptic dopamine receptors (36), but now known to consist of two enantiomers with different pre- and postsynaptic effects (30). Phenoxybenzamine pretreatment alkylates the high affinity site for 3-PPP competition at [^{3}H]dopamine-binding sites, the same site for which antipsychotic drugs have high affinity. The kainic acid-lesioning studies indicated that these high affinity antagonist sites are postsynaptic. Thus, our results do not support a presynaptic location for the [^{3}H]dopamine-binding sites for which 3-

TABLE 6

Comparison of drug dissociation constants for the antagonist high and low affinity conformations of [^{3}H]dopamine-binding sites and the concentrations of these sites

K_H and K_L represent the dissociation constants for drugs at the antagonist high and low affinity conformation of [^{3}H]dopamine-binding sites, respectively. Values are the geometric means (and 95% confidence limits) of n experiments. R_H and R_L represent the mean concentrations of binding sites (± the standard error of the mean) of antagonist high or low affinity, respectively. Values are derived from SCAFIT analysis of competition curves. The ratio of the K_H of an antagonist in competition with [^{3}H]dopamine (^{3}H·D) to the K_D of the same antagonist in competition with [^{3}H]spiroperidol (^{3}H·S) is given in the last column. The antagonists are grouped according to chemical class.

Antagonist	Drug affinities			Affinity ratios
	K_H	K_L	n	$K_H(^3H\cdot D)/K_D(^3H\cdot S)$
	nM			
Butyrophenones				
Droperidol	1.8 (0.83–3.8)	2000 (1,300–3,100)	3	2.2
Haloperidol	4.9 (4.4–5.4)	3400 (2,100–5,300)	4	7.6[a]
Lenperone	5.5 (1.8–17)	2300 (1,200–4,200)	3	0.94
Pipamperone	49 (30–81)	2900 (2,400–3,400)	3	1.7
Spiroperidol	0.91 (0.30–2.8)	2800 (2,000–4,000)	7	15[a]
Trifluperidol	1.8 (1.4–2.1)	910 (750–1,100)	4	3.6[a]
Phenothiazines				
Chlorpromazine	4.9 (3.7–6.2)	460 (230–930)	3	2.9[a]
Thioridazine	19 (14–27)	2400 (1,800–3,300)	3	5.9[a]
Trifluoperazine	15 (6.6–34)	5100 (1,900–14,000)	3	13[a]
Benzamides				
Metoclopramide	2,300 (1,400–3,900)	140,000 (93,000–220,000)	3	24[a]
Sulpiride	3,700 (2,400–5,800)	35,000 (21,000–60,000)	3	44[a]
Thioxanthines				
cis-*z*-Flupentixol	2.7 (1.7–4.2)	280 (77–980)	4	3.7[a]
Piflutixol	12 (10–13)	—[b]	3	22[a]
Other				
(+)Butaclamol	1.5 (1.3–1.7)	230 (210–260)	3	2.9[a]
(−)Butaclamol	43,000 (21,000–87,000)	—[b]	3	31[a]
Clozapine	50 (35–71)	3,200 (2,100–5,000)	4	1.0
Loxapine	9.7 (6.3–15)	700 (370–1,300)	3	2.2
Molindone	190 (130–3,000)	9,500 (6,100–15,000)	3	7.2[a]
Receptor concentrations				
R_H (fmol/mg protein)	34.3 ± 2.0			
R_L (fmol/mg protein)	32.3 ± 2.1			
n	41			

[a] Significant difference between the K_H and the K_D based on Student's t test ($p < 0.05$).

[b] —, single K_D calculated because drug has equal affinity for all [^{3}H]dopamine-binding sites.

PPP has high affinity. This does not rule out the possibility that 3-PPP may also have pharmacological actions selective for presynaptic receptors which were not measured using our receptor-binding techniques.

Effects of reserpine. In the past, reports showing that [^{3}H]dopamine binding was reduced following lesions of the substantia nigra using 6-OH-DA led to the conclusion that this loss of binding resulted from destruction of presynaptic terminals of the nigro-striatal pathway (for review, see ref. 2). Bacopoulos (37) has clearly demonstrated the error in this reasoning. Pretreatment with reserpine depleted striatal dopamine prior to receptor isolation and mimicked the reduction of [^{3}H]dopamine binding seen with the 6-OH-DA lesions. The effects of both the 6-OH-DA "lesion" and of reserpine were completely reversed by replacing the depleted dopamine during preparation of the striatal homogenate (37). These results suggest that endogenous dopamine modulates a fraction of the [^{3}H]dopamine-binding sites to enhance subsequent radioligand binding. However, the studies reported to date do not identify what component of [^{3}H]dopamine binding is regulated by dopamine itself.

In our experiments, subcutaneous pretreatment of rats with 4 mg/kg reserpine decreased binding of [^{3}H]dopamine, similar to the above report. However, it can be clearly seen (Fig. 5) that the reduction in [^{3}H]dopamine binding is restricted exclusively to the antagonist low affinity component. The reserpine pretreatment did not alter the affinity of antagonists for either their high or low affinity component or decrease the number of receptors in the antagonist high affinity conformation. Thus, [^{3}H]dopamine binds to a heterogeneous population of sites with apparent homogeneity for the radioligand. These sites have widely different affinities for most dopaminergic antagonist compounds, they are independently altered by phenoxybenzamine, reserpine, or brain lesions, and may differ in their location on striatal neural elements.

TABLE 7

Effects of phenoxybenzamine on drug affinities at [^{3}H]dopamine-binding sites

Membranes incubated with or without phenoxybenzamine were used in drug versus [^{3}H]dopamine competition assays. Data were analyzed using the SCAFIT program to determine the dissociation constants of drugs. K_L represents the dissociation constant of drugs for the low affinity component of [^{3}H]dopamine-binding sites, except for dopamine itself, which had the same dissociation constant for all these sites. Control values are geometric means from Tables 5 and 6. K_D represents the dissociation constants (with 95% confidence limits) of drugs for the apparently homogeneous population of [^{3}H]dopamine-binding sites in membranes treated with phenoxybenzamine. Phenoxybenzamine values are from experiments repeated three or more times.

Drug	Control K_L	Phenoxybenzamine (1 μM) K_D
	nM	*nM*
Dopamine	3.0 (2.6–3.5)	3.6 (2.3–5.7)
3-PPP	660 (470–910)	360 (300–450)
Droperidol	2000 (1300–3100)	1400 (780–2600)
Haloperidol	3400 (2100–5300)	1500 (1100–2100)
Spiroperidol	2800 (2000–4000)	1500 (780–2900)
Trifluperidol	910 (750–1100)	1000 (630–1600)
Chlorpromazine	460 (230–930)	280 (250–300)
(+)Butaclamol	230 (210–260)	180 (140–250)

TABLE 8

Binding of [^{3}H]spiroperidol and competitor drugs to striatal D_2 receptors using membrane preparations and assay conditions for [^{3}H]dopamine-binding experiments

K_D and B_{max} values were derived from linear or nonlinear least squares regression analysis of Scatchard plots and/or competition data, respectively. Assays were performed using [^{3}H]spiroperidol as the radioligand, but using techniques described under Materials and Methods for [^{3}H]dopamine. Values for K_D are geometric means (with 95% confidence limits). The B_{max} value is the mean (± standard error of the mean) derived from saturation and competition experiments. The number of experiments is given by *n*.

Ligand	K_D	B_{max}	*n*
	nM	*fmol/mg protein*	
[^{3}H]Spiroperidol	0.13 (0.12–0.14)[a,b]	188 ± 33	10
Haloperidol	2.0 (1.7–2.3)[a,b]		3
Lenperone	5.7 (4.6–7.2)		3
Trifluperidol	2.1 (1.7–2.6)[a]		3
Thioridazine	3.6 (3.2–4.0)[b]		3
Trifluoperazine	8.5 (8.1–9.0)[a]		3
Piflutixol	4.6 (4.5–4.8)[a,b]		3
(+)Butaclamol	2.2 (2.0–2.5)[a,b]		3
Clozapine	31 (30–33)		3
Molindone	380 (340–420)[a]		3

[a] Significant difference between the K_D obtained using [^{3}H]dopamine conditions and [^{3}H]spiroperidol conditions (Table 4), based on Student's *t* test ($p < 0.05$).

[b] Significant difference between the K_D obtained using [^{3}H]spiroperidol as the radioligand and [^{3}H]dopamine (Table 6), but under identical assay conditions, based on Student's *t* test ($p < 0.05$).

[^{3}H]Spiroperidol binding using [^{3}H]dopamine assay conditions. We were concerned that differences between the receptor isolation procedures and receptor-binding assay conditions might account for the different results we observed using [^{3}H]spiroperidol or [^{3}H]dopamine, even though the experimental conditions and membrane preparation techniques we chose were those developed by leading research groups. Therefore, experiments (summarized in Table 8) were performed using [^{3}H]spiroperidol as the ligand, but using receptor isolation and assay techniques as described above for [^{3}H]dopamine experiments. In fact, some of these experiments were executed simultaneously with [^{3}H]dopamine experiments so that the only variable was the radioligand itself. We observed that some assay-specific factors within the [^{3}H]dopamine assay were able to modify the results obtained with [^{3}H]spiroperidol, but were insufficient to account for all of the differences observed between the two radioligands. For example, [^{3}H]spiroperidol occupied approximately 188 fmol of receptors/mg of protein, regardless of the conditions utilized (*cf.* Tables 1 and 8). In contrast, [^{3}H]dopamine bound to less than 50% of this number, as reported above and by other investigators (for review, see ref. 2). In addition, the K_D of [^{3}H]spiroperidol was only slightly changed by assay conditions (Table 8), and this alteration was consistent with what could be predicted from thermodynamic effects (38).

For those competitor drugs tested (Table 8), the changes in assay conditions produced variable effects on drug potencies. For example, with haloperidol, we obtained a K_D of 2.0 nM, intermediate between the K_D values reported in Tables 4 and 6, suggesting an effect of assay conditions on the affinity of this competitor. However, assay conditions alone were insufficient to completely account for the differences in K_D values obtained in competition with the two radioligands, since the K_D we obtained (2.0 nM) was significantly different from both the K_D obtained under standard D_2 receptor assay conditions (0.64 nM, Table 4) and the K_H obtained at [^{3}H]dopamine-binding sites (4.9 nM, Table 6). However, such changes were not uniform among all competitor drugs, not even among the butyrophenones. For example, lenperone exhibited no change in affinity, regardless of the radioligand or assay conditions used (compare Tables 4, 6, and 8). For thioridazine, changing the assay conditions did not alter the K_D of this drug at D_2 receptors labeled with [^{3}H]spiroperidol (compare Tables 4 and 8), even though the K_D of this drug was 6-fold lower at [^{3}H]dopamine-binding sites (Table 6). With some other drugs, piflutixol and trifluoperazine, for example, the changes in procedures shifted the affinities to values closer to those obtained at [^{3}H]dopamine-binding sites (compare Tables 6 and 8), but significant differences between the two values were still observed (Table 8), and other competitor drugs (e.g., clozapine) did not follow the above trend.

Most important, however, was the observation that the number of binding sites labeled using [^{3}H]spiroperidol was still greatly in excess of the number occupied by [^{3}H]dopamine. In fact, the concentration of [^{3}H]spiroperidol-binding sites was the same as found for D_2 receptors earlier, regardless of membrane isolation procedures or other variables between these assays. Moreover, the binding of competitor drugs to sites labeled with [^{3}H]spiroperidol was consistent with a homogeneous population of D_2 receptors, in contrast to the complex, het-

erogeneous binding obtained using [^{3}H]dopamine as described above and illustrated in Figs. 2–5.

DISCUSSION

Our results demonstrated substantial differences between the characteristics of D_2 receptors as identified using [^{3}H]spiroperidol and those sites to which [^{3}H]dopamine binds with high affinity. The first notable difference was in the affinities of drugs for each type of site as determined from competition and saturation assays. For example, spiroperidol had very high affinity for D_2 receptors (K_D = 50–60 pM). However, in experiments studying [^{3}H]dopamine-binding sites, spiroperidol competed for two subsites with lower affinities (0.91 or 2800 nM) that both differed significantly from the affinity of the drug at D_2 receptors. Other compounds also demonstrated significant differences between their affinities at the high affinity component of [^{3}H]dopamine sites and D_2 receptors (Table 4 compared to Table 6). For haloperidol, the difference between its K_D at D_2 receptors (0.64 nM) and the high affinity component of [^{3}H]dopamine binding (4.9 nM) was approximately 8-fold (a highly significant difference). Similar differences occurred among dopaminergic antagonists of all chemical classes tested, e.g., phenothiazines, benzamides, thioxanthines, and others. In some cases, the differences in K_D values were striking (e.g., 44-fold for sulpiride, 22-fold for piflutixol). These differences also extended to several agonist compounds (compare Tables 2 and 5). Bromocriptine, for example, was 25-fold more potent at D_2 receptors than at the [^{3}H]dopamine-binding site. In contrast, dopamine itself had a 5- to 10-fold higher affinity at [^{3}H]dopamine sites (K_D = 1.4 to 3 nM) than its *highest* affinity at D_2 receptors (K_D = 16 nM).

These data differ in some ways from those of Hamblin and Creese (6, 21). Although assay conditions were chosen to be similar, the affinity of [^{3}H]dopamine appears identical, and the effects of phenoxybenzamine pretreatment are comparable; nevertheless, our conclusions differ. For example, Hamblin and Creese (21) proposed that the fraction of sites labeled with [^{3}H]dopamine for which butyrophenones had high affinity were the same sites (D_2 receptors) labeled directly with [^{3}H]spiroperidol. The K_D of spiroperidol at these [^{3}H]dopamine sites was reported as 0.27 nM, which was comparable to reports of the K_D of [^{3}H]spiroperidol in some literature sources. In similar studies using [^{3}H]apomorphine, the K_D of spiroperidol was 0.23 nM, which was compared to 0.09 nM using [^{3}H]spiroperidol (6).

Our analogous data resulted in K_D = 0.05 nM for [^{3}H]spiroperidol at D_2 receptors compared to 0.91 nM for spiroperidol at antagonist high affinity [^{3}H]dopamine-binding sites (a significant 15-fold difference). A part of the discrepancy between our data and previous reports may lie in establishing the "true" K_D of [^{3}H]spiroperidol. This ligand has been reported to have widely diverse affinities for dopamine receptors (2). Factors which affect the apparent affinity may include temperature (38) and tissue (39) or ligand concentration [causing nondopaminergic sites to be labeled (40)]. Recent reports tend toward an upper limit of affinity of [^{3}H]spiroperidol in the 50–60 pM range (3, 38, 41) as seen in our experiments. But none of the factors listed above can account for the 15-fold change which we see between spiroperidol affinity at D_2 receptors and at [^{3}H]dopamine-binding sites. In fact, when [^{3}H]spiroperidol-binding experiments were performed using experimental conditions identical to those for [^{3}H]dopamine binding, we observed only a slight increase in the K_D of [^{3}H]spiroperidol (1.5 to 2-fold) probably due to thermodynamic effects (38) and no change in the maximum number of receptors (Table 8). If variations in assay conditions were invoked as an explanation for the difference between drug affinities for D_2 receptors and for [^{3}H]dopamine-binding sites, then this factor might be expected to influence all competitor drugs, or at least those of similar chemical structures, to a comparable extent. But it can be seen from Tables 2 through 6, that certain compounds (lenperone, clozapine, etc.) have similar or identical affinities at D_2 receptors and at the antagonist high affinity site of [^{3}H]dopamine binding. Moreover, within each drug class, there is no consistent pattern to the different affinities of these drugs for D_2 receptors or [^{3}H]dopamine-binding sites. Even among the butyrophenones, no consistent effect of assay methodology could be observed. The K_D values of competitor drugs at D_2 receptors were either unchanged (lenperone), or increased about 2-fold (spiroperidol, haloperidol) or more (trifluperidol) in competition assays against [^{3}H]spiroperidol using the two methods. For some drugs (e.g., clozapine), the K_D was even decreased slightly, although not significantly, when the conditions were changed from those typical of [^{3}H]spiroperidol to those for [^{3}H]dopamine. Another compound, apomorphine, had nearly the same affinity for the agonist high affinity conformation of D_2 receptors and all [^{3}H]dopamine-binding sites, illustrating that both the differences of affinities and the similarities of affinities are found among both agonists and antagonists, and among different structural classes of antagonists. In addition, we have observed no significant differences in the affinity of either [^{3}H]spiroperidol or competitor drugs at established D_2 receptors in other brain regions (pituitary or nucleus accumbens) or striata from other species (mouse; ref. 27).[2] Therefore, it appears that the sites labeled with [^{3}H]dopamine for which neuroleptic drugs possess high affinity are not the same as D_2 receptors.

Furthermore, consider the affinity of dopamine itself. As a radioligand, its K_D is 1–2 nM (ref. 21; also Table 1). However, at the D_2 receptor, dopamine exhibited both a high and low affinity (Table 2). Its highest affinity (16 nM) is seemingly too low to conclude that it is binding to the same sites as those to which [^{3}H]dopamine binds with high affinity. However, it is known that agonist affinities can be regulated by ionic or other experimental conditions (21), thus yielding different affinities at the same receptor. Therefore, one could hypothesize that the high agonist affinity state of the D_2 receptor was the same site to which [^{3}H]dopamine was binding. In this case then, [^{3}H]dopamine should bind to about one-half as many sites as does [^{3}H]spiroperidol, since only one-half are of agonist high affinity (Table 3). Hence, we might expect [^{3}H]dopamine to bind to approximately 110

fmol of receptor/mg of protein (Table 3). All of these sites should then have a high affinity for both dopamine and spiroperidol. In fact, total [^{3}H]dopamine binding was 76 fmol/mg of protein (Table 1), significantly less than the prediction, and only one-half of these sites also had high affinity for neuroleptic drugs (34 fmol/mg of protein, Table 6).

Also, the effects of intrastriatal kainic acid lesions on [^{3}H]spiroperidol and [^{3}H]dopamine-binding sites differed. Kainic acid destroys approximately 50% of D_2 receptors (2), as also seen in our experiments. However, of the [^{3}H]dopamine-binding sites, virtually all of the antagonist high affinity receptors were destroyed by kainic acid.

A final difference between D_2 receptors and [^{3}H]dopamine-binding sites was revealed in experiments using bromocriptine. Although this drug acted as an irreversible ligand at both types of binding sites, a 25-fold higher concentration of bromocriptine was required to block irreversibly one-third of [^{3}H]dopamine-binding sites than was required to irreversibly occupy nearly one-half of D_2 receptors. This again suggests that [^{3}H]dopamine binding, even to sites with antagonist high affinity, is not to D_2 receptors as labeled with [^{3}H]spiroperidol.

The remaining sites to which [^{3}H]dopamine binds (i.e., those of antagonist low affinity) have been suggested to represent an agonist high affinity conformation of the D_1 receptor (21), since butyrophenones have a low affinity for both D_1 receptors and for this fraction of [^{3}H] dopamine-binding sites. Our results do not support this hypothesis for several reasons. Considering drug affinities first, thioxanthine drugs have been used as specific ligands for D_1 receptors (42). [^{3}H]Piflutixol binds to D_1 receptors with K_D values of 0.37 (42) to 0.86 nM[2] while [^{3}H]flupentixol binds with a K_D of 3.8 nM (42). In our experiments using [^{3}H]dopamine, the K_D of piflutixol was 12 nM, far too low an affinity to represent binding to D_1 receptors. For flupentixol, the situation is more complex. This drug, like most other antagonists, had a biphasic competition curve with high and low affinity K_D values of 2.7 and 280 nM. The high affinity K_D (2.7 nM) could indicate binding to D_1 receptors, but the alkylation of these sites by phenoxybenzamine suggests that they are similar to other high affinity antagonist sites, and therefore not D_1 receptors, which have low affinity for those same antagonists. The low affinity sites (K_D = 280 nM) have too low an affinity to represent binding to D_1 receptors. Similarly, chlorpromazine has a K_D at [^{3}H] dopamine sites of 300–460 nM in the presence or absence (low affinity site) of phenoxybenzamine. This is markedly different from its affinity at D_1 receptors (42–44). Similar comparisons with other drugs would produce comparable results. Therefore, although these antagonist low affinity [^{3}H]dopamine-binding sites possess an affinity for butyrophenones which is comparable to that of D_1 receptors, other drugs do not have similar affinities for these two sites.

Another apparent difference between these [^{3}H]dopamine-binding sites and D_1 receptors is the effect of kainic acid lesions on them. Intrastriatal kainic acid has been shown to destroy D_1 receptors almost totally, both in radioligand-binding experiments and enzymatic assays of dopamine-stimulated adenylate cyclase (2, 45). In contrast, less than total destruction of ^{3}H-agonist binding by kainic acid lesions has been reported (33, 35). We observed that one-half of [^{3}H]dopamine-binding sites were destroyed by kainic acid lesion. This destruction appeared to be limited to that fraction of sites for which spiroperidol and other antagonists have high affinity (Fig. 4). Kainic acid did not affect the remainder of sites, in contrast to its effects on D_1 receptors cited above. Therefore, it appears that [^{3}H]dopamine labels sites that are not directly comparable to either D_1 or D_2 receptors.

Our results have shown that spiroperidol binds to both D_2 receptors and [^{3}H]dopamine-binding sites. Spiroperidol has a K_D of 50–60 pM at D_2 receptors (ascertained with either labeled or nonlabeled drug) plus K_D values of 0.91 and 2800 nM for additional sites measured in competition for [^{3}H]dopamine. The extremely low affinity component is too low to be detected with direct, saturation binding techniques and, for the sake of discussion, can be ignored. However, one appropriate question would be why [^{3}H]spiroperidol appears to bind to a single class of sites in Scatchard plots when there seem to be two sites (K_D of 0.050 and 0.91 nM) that could readily be measured by direct binding techniques. The answer may

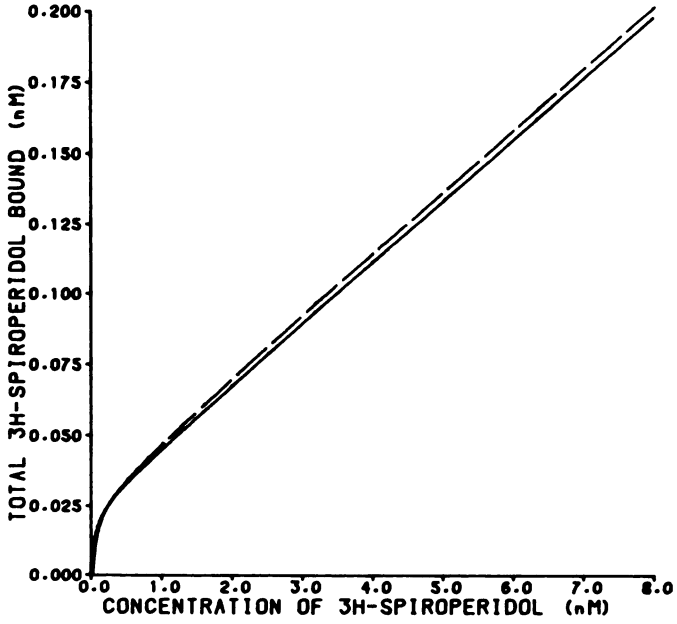

FIG. 6. *Theoretical saturation binding of [^{3}H]spiroperidol to one or more binding sites*

Computer-generated best-fit lines were developed for binding to one site (—) only, or to both the first site plus an additional site of lower concentration and lower radioligand affinity (- - -). Values chosen for the one site model were typical of those encountered in actual experiments and included a nonspecific binding component (0.225, unitless). The parameters for the one-site fit were K_D = 0.05 nM and R_T = 0.025 nM (equivalent to 200 fmol/mg of protein). The two-site model also incorporated those paramters predicted from spiroperidol competition at [^{3}H]dopamine-binding sites, e.g., K_H = 0.05 nM and K_L = 0.91 nM, R_H = 0.025 nM (200 fmol/mg of protein) and R_L = 0.0044 nM (equivalent to 35 fmol/mg of protein). These theoretical curves are virtually identical to actual data acquired when saturation isotherms of [^{3}H]spiroperidol binding were performed using 25 concentrations of ligand extending to 8 nM.

lie in the respective numbers of these sites and their relative affinities. [^{3}H]Spiroperidol labeled about 200 fmol of D_2 receptor/mg of protein, whereas the high affinity antagonist component of [^{3}H]dopamine binding was only about 35 fmol of receptor/mg of protein. Figure 6 illustrates a computer-generated best-fit line of two models, one having only one high affinity site for [^{3}H] spiroperidol as described above and the second model including additional binding to a smaller number of lower affinity sites as described above. The curves represent plots of the total (specific plus nonspecific binding) of "perfect" data. It can be seen that the lines are virtually identical over the range of usual ligand concentrations, and only diverge slightly as higher ligand concentrations are approached. Such subtle differences would never be discovered using real data because the scatter of data points around these ideal lines would mask such differences. Moreover, the error of data increases as the ligand concentration is increased. Thus, we are unable to detect more than one binding site for [^{3}H]spiroperidol using saturation binding techniques over comparable concentrations to those indicated in Fig. 6. Similarly, numerous investigators have reported an apparent homogeneity of sites labeled with [^{3}H]spiroperidol based on linear Scatchard plots, despite the fact that other methods can reveal more than one type of [^{3}H]spiroperidol-binding site (40, 46, 47).

The question yet to be resolved is to what sites or receptors these ligands are binding. Clearly, the striatal D_2 receptor, by virtue of its link to animal behavioral data and antipsychotic drug potency in humans, is an important site of neuroleptic drug action from both experimental and clinical points of view. The fact that [^{3}H] spiroperidol binds with extremely high affinity to these receptors underscores the usefulness of this ligand in dopaminergic receptor-binding assays. However, it must be recognized that under various experimental conditions it is possible to obtain binding data that suggest receptor heterogeneity. More suitable conditions using low amounts of tissue homogenate and low ligand concentrations minimize these complications. The binding of [^{3}H] dopamine, on the other hand, appears to be complex. One portion of these sites superficially resembles the D_2 receptor, whereas the other portion has some similarity to the D_1 receptor. However, sufficient differences exist to conclude that the [^{3}H]dopamine-binding sites are neither D_1 nor D_2 receptors; hence, this ligand is probably an inappropriate tool to label these receptors. Because some parallels were observed between D_2 receptors and [^{3}H]dopamine-binding sites (e.g., decreased binding after kainic acid, irreversible occupancy by bromocriptine, high affinity of neuroleptic drugs, it is possible that some relationship exists between these binding sites. For example, the antagonist high affinity component of [^{3}H] dopamine binding might reflect occupancy of an allosteric site of a larger dopamine receptor macromolecule. Recently, Bacopoulos (37) has reached a similar conclusion based upon different experimental evidence. However, until the binding of [^{3}H]dopamine is shown to be related to a pharmacological event, the utility of this ligand and the relevance of its binding sites remain to be established.

REFERENCES

1. Kebabian, J. W., and D. B. Calne. Multiple receptors for dopamine. *Nature* **277**:93–96 (1979).
2. Seeman, P. Brain dopamine receptors. *Pharmacol. Rev.* **32**:229–313 (1981).
3. Seeman, P. Commentary: nomenclature of central and peripheral dopaminergic sites and receptors. *Biochem. Pharmacol.* **31**:2563–2568 (1982).
4. Creese, I., and D. R. Sibley. Comments on the commentary by Dr. Seeman. *Biochem. Pharmacol.* **31**:2568–2569 (1982).
5. Creese, I., D. R. Burt, and S. H. Snyder. Dopamine receptor binding predicts clinical and pharamcological potencies of antipsychotic drugs. *Science* **192**:481–483 (1976).
6. Hamblin, M. W., and I. Creese. Phenoxybenzamine treatment differentiates dopaminergic ^{3}H-ligand binding sites in bovine caudate membranes. *Mol. Pharmacol.* **21**:44–51 (1982).
7. Cheng, Y. C., and W. H. Prusoff. Relationship between the inhibition constant (*Ki*) and the concentration of inhibitor which causes 50 percent of inhibition of an enzymatic reaction. *Biochem. Pharmacol.* **22**:3099–3108 (1973).
8. Hancock, A. A., A. L. DeLean, and R. J. Lefkowitz. Quantitative resolution of *beta*-adrenergic subtypes by selective ligand binding: application of a computer model-fitting technique. *Mol. Pharmacol.* **16**:1–9 (1979).
9. DeLean, A., B. F. Kilpatrick, and M. G. Caron. Dopamine receptor of the porcine anterior pituitary gland: evidence for two affinity states discriminated by both agonists and antagonists. *Mol. Pharmacol.* **22**:270–297 (1982).
10. DeLean, A., A. A. Hancock, and R. J. Lefkowitz. Validation and statistical analysis of a computer modeling method for quantitative analysis of radioligand binding data for mixtures of receptor subtypes. *Mol. Pharmacol.* **21**:5–16 (1982).
11. Hancock, A. A., and C. L. Marsh. Differences between D-2 receptors labelled with ^{3}H-spiroperidol and high affinity neuroleptic sites labelled with ^{3}H-dopamine. *Abstr. Soc. Neurosci.* **9**:1114 (1983).
12. Glowinski, J., and L. I. Iversen. Regional studies of catecholamines in the rat brain. I. The disposition of [^{3}H]norepinephrine, [^{3}H]dopamine and [^{3}H] DOPA in various regions of the brain. *J. Neurochem.* **13**:655–669 (1966).
13. Naber, D., A. Wirz-Justice, M. S. Kafka, and T. A. Wehr. Dopamine receptor binding in rat striatum: ultradian rhythm and its modification by chronic imipramine. *Psychopharmacology* **68**:1–5 (1980).
14. Braszko, J. J., M. J. Bannon, B. S. Bunney, and R. H. Roth. Intrastriatal kainic acid: acute effects on electrophysiological and biochemical measures of nigrostriatal dopaminergic activity. *J. Pharmacol. Exp. Ther.* **216**:289–293 (1981).
15. König, J. F. R., and R. A. Klippel. *The Rat Brain: A Stereotaxic Atlas.* Williams and Wilkins, Baltimore, MD, 1963.
16. Schwartz, R., K. Fuxe, L. F. Agnati, T. Hökfelt and J. T. Coyle. Rotatonal behavior in rats with unilateral striatal kainic acid lesions: a behavioral model for studies on intact dopaminergic receptors. *Brain Res.* **170**:485–495 (1979).
17. Coyle, J. T., and R. Schwarcz. Lesion of striatal neurones with kainic acid provides a model for Huntington's chorea. *Nature* **263**:244–246 (1976).
18. Bacopoulos, N. Acute change in the state of dopamine receptors: *in vitro* monitoring with ^{3}H-dopamine. *Life Sci.* **29**:2407–2414 (1981).
19. Creese, I., T. B. Usdin, and S. H. Snyder. Dopamine receptor binding regulated by guanine nucleotides. *Mol. Pharmacol.* **16**:67–76 (1979).
20. Lowry, O. H., N. J. Rosebrough, A. L. Farr, R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
21. Hamblin, M. W., and I. Creese. [^{3}H]Dopamine binding to rat striatal D-2 and D-3 sites: enhancement by magnesium and inhibition by guanine nucleotides. *Life Sci.* **30**:1587–1595 (1982).
22. Scatchard, G. The attractions of proteins for small molecules and ions. *Ann. N. Y. Acad. Sci.* **51**:660–672 (1949).
23. Hancock, A. A., and M. L. Sutterfield. *Ligand (PDP 11/70 under RSTS/E).* Biomedical Computing Technology Information Center, Nashville, TE, 1983.
24. DeLean, A., P. J. Munson, and D. Rodbard. Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose-response curves. *Am. J. Physiol.* **235**:E97–E102 (1978).
25. Bannon, M. J., A. A. Grace, B. S. Bunney, and R. H. Roth. Evidence for an irreversible interaction of bromocriptine with central dopamine receptors. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **312**:37–41 (1980).
26. Sibley, D. R., and I. Creese. Interactions of ergot alkaloids with anterior pituitary D-2 dopamine receptors. *Mol. Pharmacol.* **23**:585–593 (1983).
27. Hancock, A. A., M. W. Dudley, and N. L. Wiech. Biochemical action of RMI 11,974, a potential antipsychotic agent. *Pharmacologist* **24**:198 (1982).
28. Sibley, D. R., A. DeLean, and I. Creese. Anterior pituitary dopamine receptors: demonstration of interconvertible high and low affinity states of the D-2 dopamine receptor. *J. Biol. Chem.* **257**:6351–6361 (1982).
29. Zahniser, N. R., and P. B. Molinoff. Effect of guanine nucleotides on striatal dopamine receptors. *Nature* **275**:453–455 (1978).
30. George, S. R., M. Watanabe, and P. Seeman. The brain D_2 dopamine receptor differs from the pituitary D_2 receptor, as detected by (−)-3PPP. *Abstr. Soc. Neurosci.* **9**:31 (1983).
31. Cross, A. J., and J. L. Waddington. Kainic acid lesions dissociate [^{3}H]

spiperone and [^{3}H]cis-flupenthixol binding sites in rat striatum. *Eur. J. Pharmacol.* **71**:327–332 (1981).
32. Leff, S., L. Adams, J. Hytell, and I. Creese. Kainate lesion dissociates striatal dopamine receptor radioligand binding sites. *Eur. J. Pharmacol.* **70**:71–75 (1981).
33. Creese, I., T. Usdin, and S. H. Snyder. Guanine nucleotides distinguish between two dopamine receptors. *Nature (Lond.)* **278**:577–578 (1979).
34. Weinrich, P., and P. Seeman. Effect of kainic acid on striatal dopamine receptors. *Brain Res.* **148**:491–496 (1980).
35. Schweitzer, B. I., and N. G. Bacopoulos. Reversible decrease in dopaminergic ^{3}H-agonist binding after 6-hydroxydopamine and irreversible decrease after kainic acid. *Life Sci.* **32**:531–540 (1983).
36. Hjorth, S., A. Carlsson, H. Wikstrom, P. Lindberg, D. Sanchez, U. Hacksell, L.-E. Arvidsson, V. Svensson, and J. L. G. Nilsson, 3-PPP, a new centrally acting DA-receptor agonist with selectivity for autoreceptors. *Life Sci.* **28**:1225–1238 (1981).
37. Bacopoulos, N. G. Minireview: dopaminergic ^{3}H-agonist receptors in rat brain. New evidence on localization and pharmacology. *Life Sci.* **34**:307–315 (1984).
38. Zahniser, N. R., and P. B. Molinoff. Thermodynamic differences between agonist and antagonist interactions with binding sites for [^{3}H]spiroperidol in rat striatum. *Mol. Pharmacol.* **23**:303–309 (1983).
39. Hartley, E. J., and P. Seeman. The effect of varying ^{3}H-spiperone concentration on its binding parameters. *Life Sci.* **23**:513–518 (1978).
40. Howlett, D. R., H. Morris, and S. R. Nahorski. Anomalous properties of [^{3}H] spiperone binding sites in various areas of the rat limbic system. *Mol. Pharmacol.* **15**:506–514 (1979).
41. Hartley, E. J., and P. Seeman. Development of receptors for dopamine and noradrenaline in rat brain. *Eur. J. Pharmacol.* **91**:391–397 (1983).
42. Hytell, J. Similarities between the binding of ^{3}H-piflutixol and ^{3}H-flupentixol to rat striatal dopamine receptors *in vitro*. *Life Sci.* **28**:563–567 (1981).
43. Hytell, J. Effects of neuroleptics on ^{3}H-haloperidol and ^{3}H-cis(*Z*)-flupenthixol binding and on adenylate cyclase activity *in vitro*. *Life Sci.* **23**:551–556 (1978).
44. Cross, A., and M. Rossor. Dopamine D-1 and D-2 receptors in Huntington's disease. *Eur. J. Pharmacol.* **88**:223–229 (1983).
45. Minneman, K. P., M. Quik, and. P. C. Emson. Receptor linked cyclic AMP systems in rat neostriatum: differential localization revealed by kainic acid injection. *Brain Res.* **151**:507–521 (1978).
46. Huff, R. M., and P. B. Molinoff. Quantitative determination of dopamine receptor subtypes not linked to activation of adenylate cyclase in rat striatum. *Proc. Natl. Acad. Sci. USA* **79**:7561–7565 (1982).
47. Rosenfeld, M. R., B. Dvorkin, P. N. Klein, and M. H. Makman. Differential affinities of molindone, metoclopramide and domperidone for classes of [^{3}H] spiroperidol binding sites in rat striatum: evidence for pharmacologically distinct classes of receptors. *Brain Res.* **235**:205–211 (1982).

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