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[^{3}H]Fluphenazine Binding to Brain Membranes: Simultaneous Measurement of D-1 and D-2 Receptor Sites

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Abstract: [^{3}H]Fluphenazine was used to label both D-1 and D-2 dopamine receptors in mouse striatal membranes. The D-1 and D-2 specific binding of [^{3}H]fluphenazine was discriminated by the dopamine antagonists SCH-23390 (D-1 selective) and spiperone (D-2 selective). Saturation analyses of these two sites yielded a D-1 receptor density in mouse striatum of 1,400 fmol/mg of protein and a D-2 receptor density of 700 fmol/mg of protein. The affinity of [^{3}H]fluphenazine for the D-2 site was slightly greater than for the D-1 site; the equilibrium dissociation constant (K_D) was 0.7 versus 3.2 n*M*, respectively. Assay conditions are described that reduce nonspecific binding of [^{3}H]fluphenazine to acceptable levels (35% of total binding at 1 n*M* [^{3}H]fluphenazine). By comparison of displacement curves from a series of dopaminergic and nondopaminergic ligands, the pharmacological specificity of [^{3}H]fluphenazine binding in mouse striatum was demonstrated to be dopaminergic. Only small amounts of dopamine-specific (apomorphine-sensitive) [^{3}H]fluphenazine binding were found in other brain regions. However, chlorpromazine displaced considerable [^{3}H]fluphenazine from all brain regions, including cerebellum, suggesting the presence of a [^{3}H]fluphenazine binding site with a phenothiazine specificity. **Key Words:** Dopamine receptors — [^{3}H]Fluphenazine — Spiperone — SCH-23390—Striatum. **Morgan D. G. and Finch C. E.** [^{3}H]Fluphenazine binding to brain membranes: Simultaneous measurement of D-1 and D-2 receptor sites. *J. Neurochem.* **46**, 1623–1631 (1986).

Dopamine binding sites in mammals consist of at least two types, designated D-1 and D-2 (Kebabian and Calne, 1979; Stoof and Kebabian, 1984). This classification was originally based on the differentiation of two dopamine receptors, one activating adenylate cyclase (D-1) and the other not linked to adenylate cyclase (D-2). However, recent evidence suggests that the D-2 receptor may actually inhibit adenylate cyclase (Stoof and Kebabian, 1981; Cronin and Thorner, 1982; Frey et al., 1982). The parathyroid gland is one tissue that contains only D-1 receptors (Cross et al., 1983), whereas the pituitary gland contains only D-2 receptors (DeLean et al., 1982; Sibley et al., 1982*a*). Both classes of dopamine binding sites are found in striatum. Recent data indicate that the D-1-selective agonists and antagonists produce a behavioral profile similar to D-2-selective and nonselective dopaminergic drugs when injected in vivo, indicating both striatal binding sites are authentic dopamine receptors (Arnt and Hyttel, 1984; Christensen et al., 1984; Mailman et al., 1984).

The molecular pharmacology of the D-2 site has been extensively described using several ^{3}H-ligands (Seeman, 1980). The D-1 binding site has been studied using the dopamine antagonists [^{3}H]flupentixol (Hyttel, 1978; Cross and Owen, 1980; Leff et al., 1981; Murrin, 1983; Huff and Molinoff, 1985) or [^{3}H]piflutixol (Hyttel, 1981; Cross et al., 1983; O'Boyle and Waddington, 1984). Because these thioxanthine ligands bind to both D-1 and D-2 receptors with approximately equal affinity, accurate measurement of D-1-specific binding characteristics requires occlusion of the D-2 site by a nonlabeled ligand with at least a 1,000-fold greater affinity for the D-2 site. The widespread use of these radioligands has been limited by high levels of nonspecific binding and low specific activity, necessi-

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Abbreviations used: BSA, bovine serum albumin; dopamine, 3,4-dihydroxyphenylethylamine; LSD, D-lysergic acid diethylamide; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PEI, polyethyleneimine; serotonin, 5-hydroxytryptamine.

tating the use of large tissue quantities. Most recently, [^{3}H]SCH-23390 was briefly described as a D-1-selective ligand (Schulz et al., 1984).

Radiolabeled dopamine agonists also label dopamine receptors. However, only the fraction of the receptors in the high-agonist-affinity conformation are measured using ^{3}H-agonists (Grigoriadis and Seeman, 1983; Hamblin et al., 1984). Moreover, both D-1 and D-2 receptors have high-agonist-affinity states that are labeled by ^{3}H-agonists, necessitating discrimination of D-1 and D-2 receptor specific binding, as described here for [^{3}H]fluphenazine. The fraction of dopamine receptors in the high-agonist-affinity state can be modulated by a number of in vitro parameters, such as ionic conditions, incubation temperature, guanyl nucleotides, and the amount of dopamine present in the homogenate (Creese et al., 1984). Given the fact that ^{3}H-agonists label only a portion of the receptors and that this fraction is sensitive to a variety of in vitro parameters, we believe that widespread use of ^{3}H-agonists to quantify dopamine receptor density will require more detailed understanding of the formation and functional significance of the high-agonist-affinity state of D-1 and D-2 dopamine receptors.

We report here the characterization of another ^{3}H-ligand for use in quantitating D-1 binding sites. The major advantage of [^{3}H]fluphenazine over [^{3}H]flupentixol or [^{3}H]piflutixol is its fivefold greater specific activity (as presently available) and, under the conditions described here, its considerably lower nonspecific binding. [^{3}H]Fluphenazine also has a greater specific activity than [^{3}H]SCH-23390. Neither [^{3}H]piflutixol nor [^{3}H]SCH-23390 is commercially available. When [^{3}H]fluphenazine is combined with appropriate concentrations of D-1- or D-2-selective competitors, the binding of [^{3}H]fluphenazine to D-1 and D-2 sites can be discriminated and the characteristics of each site can be measured in a single assay.

EXPERIMENTAL PROCEDURES

Materials

[^{3}H]Fluphenazine (specific activity 37–39 Ci/mmol) was purchased from New England Nuclear and stored at −20°C under argon for up to 3 months. 3-(*N*-Morpholino)propanesulfonic acid (MOPS) and other chemicals were purchased from Sigma.

Several classes of drugs were tested for their capacity to displace [^{3}H]fluphenazine from striatal membranes: the phenothiazines fluphenazine (Squibb), chlorpromazine (Smith, Klein, & French), and perphenazine (Schering); the butyrophenones domperidone and spiperone (both from Janssen); the substituted benzamide sulpiride (racemate; Adria); the hallucinogen D-lysergic acid diethylamide (LSD: National Institute on Drug Abuse); the serotonin S-2 receptor antagonists ketanserin (Janssen) and LY-53857 (Lilly); the α_1-adrenergic receptor antagonist prazosin (Pfizer); the α_2-adrenergic receptor antagonist yohimbine (Sigma); the β-adrenergic receptor antagonist propranolol (Sigma); the putative neurotransmitters dopamine, epinephrine, norepinephrine, serotonin, histamine, and adenosine (all from Sigma); and the psychoactive drugs apomorphine, imipramine (both from Sigma), and morphine (National Institute on Drug Abuse).

Preparation of membranes

Striata from 2–4-month-old C57BL/6J or Swiss-Webster male mice were dissected, frozen on dry ice, and stored at −70°C until assayed. Frozen striata were homogenized in 50 volumes of cold 20 m*M* MOPS/Tris, 4 m*M* $MgSO_4$, and 1 m*M* EDTA (pH 7.3) with a Polytron at a setting of 5 for 10 s and incubated at 37°C for 15 min in a shaking water bath. Homogenates were cooled by addition of another 50 volumes of cold homogenization buffer in an ice bath and then centrifuged at 48,000 *g* for 10 min at 4°C. The pellet was resuspended with use of a Polytron at a setting of 5 for 5 s in 50 volumes of fresh homogenization buffer, diluted with another 50 volumes, and centrifuged once more. The membrane-containing final pellet was resuspended with use of a Polytron at a setting of 5 for 5 s in 50 volumes of cold homogenization buffer, diluted with another 30 volumes, and added to assay tubes. A new membrane pool was prepared for each assay.

Binding assay

[^{3}H]Fluphenazine binding is assayed under conditions similar to those used for [^{3}H]spiperone labeling of D-2 binding sites (Creese et al., 1977; Morgan et al., 1984*a*; Seeman et al., 1984). Except where indicated, assays were performed in disposable glass test tubes to which 500 μl of assay buffer (double strength), 100 μl of competing drugs (in 0.067% ascorbic acid), 200 μl of [^{3}H]fluphenazine (in 0.067% ascorbic acid), and 200 μl of membrane suspension (in homogenization buffer) were added sequentially. Final concentrations in the assay tubes were 24 m*M* MOPS/Tris, 100 m*M* NaCl, 5 m*M* KCl, 2 m*M* $CaCl_2$, 2 m*M* $MgSO_4$, 0.02% ascorbic acid, 0.2 m*M* EDTA (pH 7.2), and 150–250 μg of membrane protein. In some instances, 0.1% bovine serum albumin (BSA) was present in all assay tubes (see figure legends and table footnotes). Assays were initiated by addition of the membrane suspension and incubated for 30 min at 25°C. Assays were quenched by addition of 5 ml of cold wash buffer (2 m*M* MOPS/Tris and 150 m*M* NaCl, pH 7.3) and filtered through Whatman GF/C filters [wetted briefly with 0.1% polyethyleneimine (PEI)] using a 24-port cell harvester (Brandel, Gaithersburg, MD, U.S.A.) and a 150-cfm vacuum pump. Filters were rinsed four times with 5 ml of wash buffer. The entire filtration procedure was completed within 20 s. Radioactivity bound to the filters was counted by liquid scintillation spectroscopy at 35% efficiency.

The peroxidation of membrane lipids by ascorbic acid, as monitored by malonyl dialdehyde formation, did not occur using these assay conditions (P. C. May, D. Salo, D. G. Morgan, and C. E. Finch, submitted). This was attributed to chelation of iron by EDTA in the homogenization-centrifugation buffers, because omitting EDTA resulted in measureable malonyl dialdehyde formation.

Membrane protein content was estimated with the Coomassie Brilliant Blue dye binding reagent (Bio-Rad, Richmond, CA, U.S.A.) following solubilization of membrane proteins in 1 *M* NaOH for 30 min at room temperature.

RESULTS

Time course

The association of [^{3}H]fluphenazine with mouse striatal membranes is shown in Fig. 1. Association was essentially complete by 30 min at 25°C and remained at equilibrium until 90 min. The 30-min incubation time was used for subsequent assays because longer incubations would increase the possibility of ascorbic acid-induced membrane peroxidation (Heikkila et al., 1983), oxidation of some competitors such as catechols and indoles, or proteolysis of membrane proteins. Addition of apomorphine (final concentration 100 μM) to the incubation at 30 min revealed a time-dependent dissociation of [^{3}H]fluphenazine from the membrane, indicating reversible binding with a $t_{1/2}$ of 6.9 min. The affinity constant (K_D), estimated from the on rate and off rate (k_{-1}/k_{+1}; Bylund, 1981), was 1.02 nM, which is close to the aggregate K_D estimated from equilibrium studies (see Table 5). The linearized off rate (ln B/B_0 versus time) was slightly curvilinear, suggesting site heterogeneity, as expected for a ligand binding to at least two sites.

Minimization of nonspecific binding

Our initial characterization of [^{3}H]fluphenazine binding to mouse striatal membranes was hindered by an unfavorable ratio of specific to nonspecific binding. Under assay conditions similar to those used for [^{3}H]spiperone labeling of D-2 binding sites (Morgan et al., 1984*a*), ~35% of the binding was displaced by 100 μM apomorphine. To reduce nonspecific binding, we tested the influence of (a) adding 0.1% BSA to the assay buffer, (b) using 0.1% PEI to wet the glass fiber filters before filtration, and (c) adding 150 mM NaCl to the wash buffer. The addition of NaCl to the wash buffer was the most effective treatment for reducing nonspecific binding (Table 1). Both the PEI treatment and the BSA in the assay reduced nonspecific binding as well; however, the effect of BSA in the assay was not additive with the PEI filter wetting. The combination of NaCl wash and PEI filter wetting gave 65% specific binding at 1 nM [^{3}H]fluphenazine and 150 μg of membrane protein. At higher protein concentrations, the percentage of specific binding was increased proportionately. With 250 μg of protein in saturation analyses, specific binding exceeded 50% at 16 nM [^{3}H]fluphenazine (the highest concentration studied).

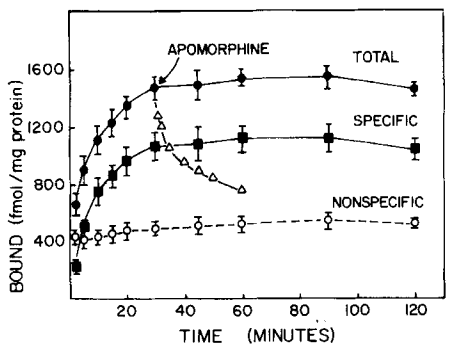

FIG. 1. Time course of [^{3}H]fluphenazine (1.8 nM) binding to mouse striatal membranes. Assays were initiated by addition of 200 μl of membrane suspension to assay tubes (on ice), and tubes were placed in a 25°C water bath. Assay tubes were filtered at the times indicated. In some instances, apomorphine (100 μl; final concentration 100 μM) was added at 30 min of incubation (equilibrium), and the dissociation rate of [^{3}H]fluphenazine was followed. Data are mean ± SEM (bars) values from three experiments, each performed in quadruplicate. BSA was not used in these assays. Nonspecific binding was estimated with 100 μM apomorphine.

Choice of competitor for nonspecific binding

Comparison of displacement curves produced by dopaminergic ligands revealed that some ligands displaced more binding than others. In general, dopamine antagonists displaced [^{3}H]fluphenazine to a greater extent than dopamine agonists or nondopaminergic ligands (LSD and ketanserin). Apomorphine displacement curves ended with a broad plateau region, and apomorphine failed to displace [^{3}H]fluphenazine from boiled membranes even at 1 mM (Fig. 2). Chlorpromazine (10 μM), a ligand used by others to estimate nonspecific binding (Freedman et al., 1983), displaced more [^{3}H]fluphenazine than apomorphine or other agonists (to 15% of the total incorporation), but it also displaced [^{3}H]fluphenazine from boiled membranes (Fig. 2). In addition, chlorpromazine, but not apomorphine, displaced [^{3}H]fluphenazine from cerebellar membranes (see Table 4). Because neither dopamine nor dopamine receptors have been reported in cerebellum (Seeman, 1980; Lindvall and Bjorklund, 1983), it would appear that chlorpromazine can displace [^{3}H]fluphenazine from nondopaminergic sites. Because the identity of this chlorpromazine-sensitive, apomorphine-insensitive [^{3}H]fluphenazine binding site is not known (see Discussion), we

TABLE 1. *Nonspecific binding of [^{3}H]fluphenazine under various assay conditions*

Condition	Mean cpm	SEM	Percentage of control
Control	4,050	80	100
BSA	3,330	80	82
PEI	2,260	25	56
NaCl wash	1,840	30	46
BSA + PEI	2,390	40	59
BSA + NaCl wash	1,500	20	37
PEI + NaCl wash	1,470	7	36
BSA + PEI + NaCl wash	1,450	30	36

Assay tubes contained 1 nM [^{3}H]fluphenazine, 150 μg of striatal membrane protein, and 100 μM apomorphine to estimate nonspecific binding. Specific binding (~2,500 cpm) did not change appreciably under these conditions. Results presented here are the average and SEM of two experiments performed in triplicate. BSA was 0.1% in the assay tubes, PEI was 0.1% on the filters immediately before filtration, and NaCl wash indicates 0.9% NaCl added to the wash buffer.

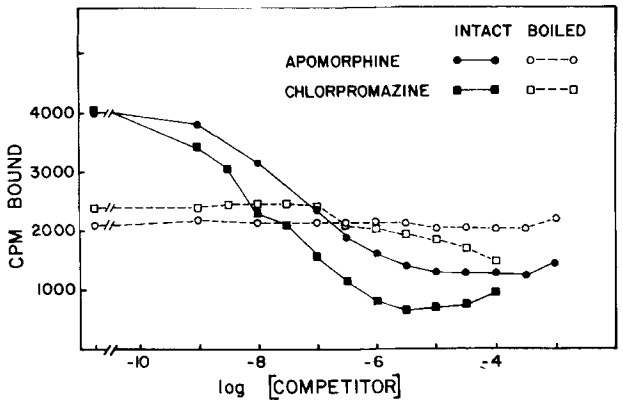

FIG. 2. Displacement of [^{3}H]fluphenazine (1.5 n*M*) from intact and boiled mouse striatal membranes. One-half of striatal membrane preparation was placed in a boiling water bath for 30 min, cooled in an ice bath, and resuspended by homogenization with a Polytron at a setting of 5 for 5 s. The other half was incubated in parallel on ice and homogenized like the boiled material. Each preparation was then assayed for [^{3}H]fluphenazine binding as described in Experimental Procedures with 0.1% BSA present in all tubes. The results are presented as total binding, rather than specific binding, to demonstrate differences in the estimated levels of nonspecific binding. The data presented here are from a single study representative of two experiments performed in triplicate. SE values were <10% of the average value in all cases.

used 100 μ*M* apomorphine to estimate nonspecific binding in [^{3}H]fluphenazine assays.

Pharmacological specificity of [^{3}H]fluphenazine binding to striatal membranes

The pharmacological specificity of [^{3}H]fluphenazine binding was established by testing the capacity of several classes of ligands to displace [^{3}H]fluphenazine binding (Tables 2 and 3). Dopamine was the most potent monoamine transmitter in displacing [^{3}H]fluphenazine binding. Dopaminergic antagonists were more potent than serotonergic antagonists in displacing [^{3}H]fluphenazine, whereas adrenergic antagonists were ineffective at displacing [^{3}H]fluphenazine from striatal membranes.

Some dopamine antagonists produced multiphasic displacement curves and fitted a two-site model significantly better than a one-site model, as assessed by the computer program LIGAND (Munson and Rodbard, 1980; Table 3). Of these compounds, spiperone and domperidone exhibited the greatest discrimination of the two sites, with 2,400- and 3,200-fold differences, respectively, in affinity. Although domperidone produced slightly better discrimination of D-1 and D-2 components, its 10-fold lower absolute affinity for these sites, relative to spiperone, increased the possibility of nonspecific displacement of [^{3}H]fluphenazine, as observed for chlorpromazine (see above). With the exception of SCH-23390, the ligands in Table 3 are reported to be selective for the D-2 site (Seeman, 1980), indicated as the "high-affinity site" in Table 3.

The dopamine antagonist SCH-23390 is reported to be selective for the D-1 dopamine receptor (Hyttel, 1983). Consistent with such a property, SCH-23390 displaced [^{3}H]fluphenazine from putative D-1 and D-2 components with >1,000-fold selectivity (Table 3 and Fig. 3A). Spiperone produced similar displacements with an intermediate plateau (Fig. 3B). To determine whether the high- and low-affinity components of the SCH-23390 and spiperone displacement curves were the same components or whether they were inverted, displacement curves for one competitor were performed in the presence of a constant concentration of the other competitor that occluded the high-affinity component (Fig. 3). Addition of 10 n*M* spiperone prevented [^{3}H]fluphenazine binding to the low-affinity component of the SCH-23390 displacement curve, suggesting this is the D-2 component. Similarly, addition of 10 n*M* SCH-23390 prevented [^{3}H]fluphenazine binding to the low-affinity component of the spiperone displacement curve. This complementation indicates that the high-affinity components of the SCH-23390 and spiperone displacement curves are *not* identical and correspond to the D-1 and D-2 dopamine receptors, respectively. Thus, to measure [^{3}H]fluphenazine binding to only the D-1 site, an intermediate concentration of spiperone was included in all assay tubes to occlude the D-2 component (see columns headed D-1 in Tables 2 and 3).

Regional specificity of [^{3}H]fluphenazine binding to brain membranes

[^{3}H]Fluphenazine binding was compared in four mouse brain regions (Table 4). Specific binding was greatest in striatum, followed by whole cerebral cortex (~15% of striatum) and hippocampus. No apomorphine-displaceable binding was found in cerebellum. Nonspecific binding, estimated with 100 μ*M* apomorphine, was twice as high in cerebellum as in other brain regions. Chlorpromazine consistently displaced more [^{3}H]fluphenazine binding than apomorphine, especially in cerebellum.

The pharmacological specificity of [^{3}H]fluphenazine binding in these four regions was tested with 10 n*M* SCH-23390 (D-1 selective), 10 n*M* spiperone (D-2 selective), 100 n*M* ketanserin (S-2 selective), and 10 n*M* prazosin (α_1-adrenergic selective). In striatum, [^{3}H]fluphenazine binding was primarily dopaminergic in nature, with approximately equal amounts of D-1 and D-2 binding at this [^{3}H]fluphenazine concentration: a small serotonergic component (6% of the total) was also detected. In cerebral cortex, there was evidence of [^{3}H]fluphenazine binding to both serotonergic and adrenergic sites, although displaceable sites were few in comparison to striatum. In hippocampus and cerebellum, little

TABLE 2. *Displacement of [³H]fluphenazine binding to mouse striatal membranes by drugs that fail to discriminate D-1 and D-2 binding sites*

	Aggregate[a] K_i (n*M*) (D-1 + D-2)	D-1[a] K_i (n*M*)	n
Dopamine antagonists			
Chlorpromazine	6.4 ± 0.4	13 ± 1	3
Perphenazine	1.8 ± 0.4	3.2 ± 0.5	3
Fluphenazine	1.0 ± 0.3	1.9 ± 0.3	3
Haloperidol	9.4 ± 0.5	20.4 ± 2	3
Serotonin antagonists			
LSD	208 ± 100	294 ± 60	3
Ketanserin	530 ± 45	507 ± 170	3
LY-53857	276 ± 86	105 ± 9	2
Adrenergic antagonists			
Prazosin	>1,000	>1,000	3
Yohimbine	>1,000	>1,000	3
Propranolol	>1,000	>1,000	3
Agonists[b]			
Apomorphine	252 ± 51	—	5
Dopamine	4259 ± 840	2,680 ± 850	7
Epinephrine	7,800 ± 1,000	7,580 ± 1,870	3
Norepinephrine	13,700 ± 4,180	15,600 ± 5,000	3
Serotonin	21,062 ± 6,000	49,200 ± 9,100	3
Imipramine	591 ± 15	711 ± 20	2
Histamine	>10,000	>10,000	3
Adenosine	>10,000	>10,000	3
Morphine	>10,000	>10,000	2

Data are mean ± SEM values where indicated.

[a] For aggregate displacements, 1.5 n*M* [³H]fluphenazine was used with 100 μ*M* apomorphine to estimate nonspecific binding. All compounds in Table 2 did not fit the two-site model significantly better than the one-site model using the program LIGAND (Munson and Rodbard, 1980) on the Apple II+ computer. Thus, K_i values for the combined D-1 plus D-2 sites are presented for the "Aggregate" condition. To measure displacement exclusively from the D-1 binding site, 30 n*M* spiperone was added to all tubes to mask the D-2 site, and the [³H]fluphenazine concentration was increased to 3 n*M* (approximate K_D of the D-1 site). The affinities of the "labeled" ligand for use in the computer program were constrained to the values obtained from saturation analyses of [³H]fluphenazine binding (see Table 5). Each compound was tested at 12 concentrations spanning at least four orders of magnitude. All assay tubes contained ~150 μg of striatal membrane protein and 0.1% BSA and were performed in triplicate.

[b] For agonist displacement curves, the LIGAND program was restricted to the single-site model. Theoretically, agonists should have four affinities for the aggregate binding sites: high and low D-2 (Hamblin et al., 1984; Wreggett and Seeman, 1984) and high and low D-1 (Sibley et al., 1982*b*). Accurate determination of these affinities will require the use of selective D-1 and D-2 blocking drugs, plus the use of guanyl nucleotides/divalent cations to modulate the high/low-affinity states of these binding sites. The present analysis was designed to determine which agonist has the greatest overall affinity for the aggregate and D-1 binding sites.

displacement of [³H]fluphenazine was produced by any of these ligands, indicating negligible specific binding in these regions.

Attempts at saturation analyses of [³H]fluphenazine binding to nonstriatal tissues were unsuccessful because of low levels of specific binding (using 100 μ*M* apomorphine as blank).

Density of D-1 and D-2 binding sites in mouse striatum

In saturation analyses of [³H]fluphenazine binding to mouse striatal membranes, the D-1 and D-2 components were quantitated simultaneously. This was accomplished by assaying total binding tubes, nonspecific binding tubes (containing 100 μ*M* apomorphine), and a tube containing intermediate spiperone or SCH-23390 concentrations to discriminate D-1 and D-2 specific binding. The spiperone or SCH-23390 concentration was covaried with [³H]fluphenazine concentration to account for shifts in the plateau region of the spiperone displacement curve at different [³H]fluphenazine concentrations (Boehme and Ciaranello, 1982). This design allowed three saturation curves to be measured: the total or aggregate specific binding (total minus nonspecific), the D-2-specific

TABLE 3. *Displacement of [³H]fluphenazine binding to mouse striatal membranes by drugs that discriminate D-1 and D-2 binding sites*

Dopamine antagonist	Aggregate[a] K_i (n*M*) high-affinity site (D-2)	Aggregate[a] K_i (n*M*) low-affinity site (D-1)	K_i ratio D-1/D-2	D-1[a] K_i (n*M*)	n[b]
Spiperone	0.086 ± 0.011	205 ± 36	2,400	—	8
Domperidone	0.82 ± 0.20	2,700 ± 1,000	3,200	3,800 ± 1,000	4
Sulpiride	19.4 ± 4.4	16,000 ± 2,400	820	10,900 ± 1,600	4
Pimozide	0.51 ± 0.20	450 ± 150	880	332 ± 35	3
SCH-23390	245 ± 50	0.13 ± 0.04	1/1,900	0.20 ± 0.08	3

Data are mean ± SEM values as indicated.

[a] Assays were conducted essentially as described in Table 2. The "aggregate" displacement curves produced by these compounds fit a two-site model significantly better than a one-site model using the computer program LIGAND (Munson and Rodbard, 1980). The component with high affinity for *most* competitors was the D-2 binding site. This site had approximately half the receptor concentration of the low-affinity component. For determination of the D-1 K_i, D-2 sites were occluded with 30 n*M* spiperone (3 n*M* [³H]fluphenazine), and data were analyzed using the one-site model.

[b] Number of experiments.

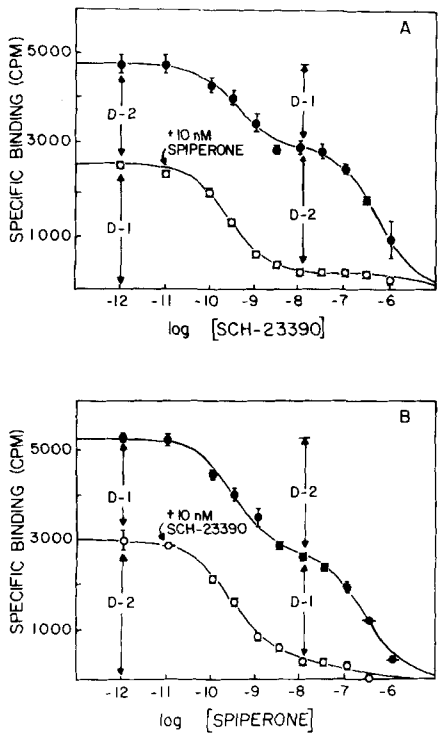

FIG. 3. Displacement of [³H]fluphenazine (2 n*M*) by SCH-23390 and spiperone. **A:** [³H]Fluphenazine binding to striatal membranes was competed against by increasing concentrations of SCH-23390 in the absence (●) or presence (○) of 10 n*M* spiperone. **B:** The converse experiment is presented with increasing concentrations of spiperone in the absence (●) or presence (○) of 10 n*M* SCH-23390. For both ligands, 10 n*M* is sufficient to occlude the high-affinity component of the displacement curve. The portions of each curve tentatively identified as D-1- or D-2-specific binding are indicated. The lines represent the best fit for each curve using parameters derived from LIGAND (Munson and Rodbard, 1980). Data are given as specific binding using 100 μ*M* apomorphine to estimate nonspecific binding. Data are mean ± SEM (bars) values from a single study representative of four experiments performed in quadruplicate. No BSA was present in these assays.

binding (total minus intermediate spiperone, or intermediate SCH-23390 minus nonspecific), and the D-1-specific binding (spiperone minus nonspecific, or total minus SCH-23390). The Scatchard plots derived from such an analysis using spiperone to discriminate D-1 and D-2 components are presented in Fig. 4.

Similar B_{max} and K_D values were obtained irrespective of the ligand used to discriminate the D-1 and D-2 components of [³H]fluphenazine binding (either spiperone or SCH-23390; Table 5). Moreover, nonspecific binding values were the same using apomorphine (100 μ*M*) or spiperone plus SCH-23390 (each at intermediate concentrations). When using 10 μ*M* chlorpromazine to estimate nonspecific binding, it consistently displaced 15–25% more [³H]fluphenazine than apomorphine and typically produced linear Scatchard plots, but with higher K_D and B_{max} values. The density and affinity values obtained by these different methods are compared in Table 5.

DISCUSSION

A critical decision in characterizing [³H]fluphenazine binding is the choice of the appropriate compound to use for estimating nonspecific binding. Several hydrophobic antagonist ligands displace 15–25% more total binding than the agonist ligands dopamine or apomorphine. We thought, a priori, that any antagonist binding not displaceable by neurotransmitter agonists probably does not represent a physiological receptor for that neurotransmitter. Moreover, the dopamine antagonist chlorpromazine displaces [³H]fluphenazine from boiled membranes and cerebellar membranes; in contrast, apomorphine does not. However, because boiled membranes have more [³H]fluphenazine bound than the nonspecific binding of intact membranes, it is conceivable that the chlorpromazine-sensitive,

TABLE 4. *Displacement of [³H]fluphenazine binding by selective antagonists in four regions of mouse brain*

A. Binding	fmol/mg of protein			
	Striatum	Cortex	Hippocampus	Cerebellum
Specific	1,134 ± 62	159 ± 10	83 ± 10	−24 ± 11
Total	1,816 ± 97	743 ± 58	703 ± 48	1,360 ± 107
Apomorphine (10^{-4} *M*)	681 ± 36	584 ± 58	620 ± 44	1,384 ± 96
Chlorpromazine (10^{-5} *M*)	282 ± 13	197 ± 9	249 ± 8	292 ± 11
	Percentage of total			
B. Displacer	Striatum	Cortex	Hippocampus	Cerebellum
SCH-23390 (10^{-8} *M*)	30 ± 3	5 ± 3	2 ± 1	−4 ± 3
Spiperone (10^{-8} *M*)	27 ± 3	12 ± 1	3 ± 1	0 ± 2
Ketanserin (10^{-7} *M*)	6 ± 2	14 ± 1	5 ± 1	0 ± 3
Prazosin (10^{-8} *M*)	−1 ± 1	8 ± 1	3 ± 1	3 ± 2

Data in Table 4A are the amounts of [³H]fluphenazine bound. Specific binding is the difference between total binding and that remaining in the presence of apomorphine. Data in Table 4B are percentages of the total binding displaced by each ligand. D-1 receptor-specific binding was estimated by adding SCH-23390, D-2-specific binding by adding spiperone, S-2 serotonin receptor binding by adding ketanserin, and α_1-adrenergic receptor binding by adding prazosin. The [³H]fluphenazine concentration for these three experiments was 2.8 ± 0.2 n*M*. Results are the average ± SEM of three experiments performed in triplicate.

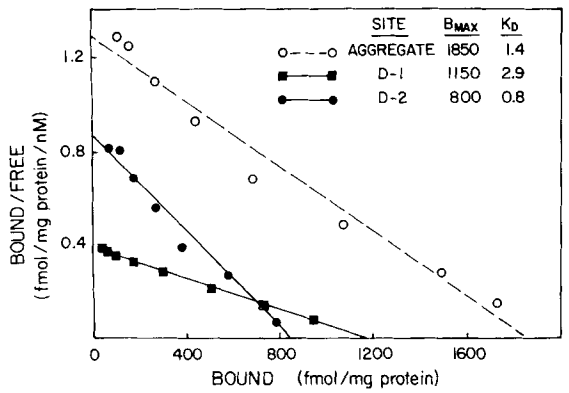

FIG. 4. Saturation analysis of [³H]fluphenazine binding (0.1–16 n*M*) to D-1, D-2, and aggregate (total) components in mouse striatal membranes. The Scatchard plots of D-1, D-2, and aggregate specific binding are presented. The three components were quantitated simultaneously using a total binding tube (no additions), a tube containing an intermediate spiperone concentration (to occlude D-2-specific binding), and a tube containing 100 μ*M* apomorphine (nonspecific binding or blank tube). The [³H]fluphenazine concentrations were 0.12, 0.25, 0.5, 1, 2, 4, 8, and 16 n*M*. The spiperone concentration was covaried with the [³H]fluphenazine concentration at 10 times the [³H]fluphenazine concentration (1.2–160 n*M*). The difference between the total binding and nonspecific binding (apomorphine-containing) tubes at each concentration was used for the aggregate plot. The D-2-specific binding was the difference between total binding and the binding remaining in the spiperone tube (total − spiperone); D-1-specific binding was the difference between the spiperone-containing tube and nonspecific binding (spiperone − apomorphine). The present results are derived from quadruplicate determinations. Lines are drawn from linear regression analyses with r^2 values in excess of 0.95. We have performed >50 saturation analyses using mouse brain membranes, some of which are summarized in Table 5.

apomorphine-insensitive displacement is from sites created by boiling. Thus, although not conclusive, these results imply that the chlorpromazine-sensitive, apomorphine-insensitive binding is nonspecific-displaceable binding. Others have reported similar results, e.g., butaclamol displacement of [³H]flupentixol from glass fiber filters (no membranes present; Murrin 1983). This artifactual binding "site" may be analogous to the [³H]fluphenazine binding displaced from boiled membranes. However, saturation analyses indicated a saturable component of [³H]fluphenazine binding that was insensitive to apomorphine and displaced by chlorpromazine (Table 5; "PTZ? site"). Hence, there may be a nondopaminergic "phenothiazine site" of unknown specificity or function. In any case, these results typify the pitfalls of using compounds to estimate nonspecific binding that are similar in chemical structure to the radioligand used in the receptor binding assay.

Additional support for the use of apomorphine to estimate nonspecific binding derives from the [³H]fluphenazine displacement by SCH-23390 and spiperone. The displacement curves of these two dopamine antagonists are both biphasic and model two sites with >1,000-fold differences in affinity. The high-affinity components of these curves do not correspond to the same binding site and, presumably, represent the D-1 binding site (SCH-23390) and the D-2 binding site (spiperone). The sum of the high-affinity components of these two competitors approximates the total displacement by apomorphine (Fig. 3; see also Table 5). This is less than the displacement obtained with chlorpromazine (Fig. 2 and Table 5).

Saturation analyses of [³H]fluphenazine binding could be resolved into two components using SCH-23390 or spiperone to discriminate binding to

TABLE 5. *Comparison of different methods for discriminating D-1 and D-2 components in saturation analyses of [³H]fluphenazine binding to D1, D2, aggregate, and PTZ? sites in mouse striatal membranes*

Total	Blank	B_{max}	K_D	r^2
Aggregate site				
Total	Apomorphine	1,870 ± 130	1.55 ± 0.24	0.98
Total	Spiperone + SCH-23390	1,910 ± 220	1.28 ± 0.34	0.94
Total	Chlorpromazine	2,540 ± 150	1.97 ± 0.22	0.98
D-1 site				
Total	SCH-23390	1,450 ± 110	3.64 ± 0.73	0.94
Spiperone	Apomorphine	1,220 ± 140	2.98 ± 0.22	0.96
Spiperone	Spiperone + SCH-23390	1,370 ± 230	3.09 ± 0.36	0.96
Spiperone	Chlorpromazine	1,980 ± 180	3.65 ± 0.25	0.98
D-2 site				
Total	Spiperone	780 ± 50	0.79 ± 0.20	0.94
SCH-23390	Apomorphine	700 ± 50	0.74 ± 0.14	0.96
SCH-23390	Spiperone + SCH-23390	705 ± 70	0.66 ± 0.16	0.98
SCH-23390	Chlorpromazine	1,280 ± 160	1.20 ± 0.22	0.96
PTZ? site				
Apomorphine	Chlorpromazine	985 ± 130	6.70 ± 1.10	0.91

Saturation analyses were performed using eight concentrations of [³H]fluphenazine (0.12, 0.25, 0.5, 1, 2, 4, 8, and 16 n*M*). At each concentration, the following competitors were used: none (total binding), spiperone (10 × [³H]fluphenazine concentration), SCH-23390 (10 × [³H]fluphenazine concentration), spiperone plus SCH-23390, apomorphine (100 μ*M*), and chlorpromazine (10 μ*M*). A series of Scatchard plots were constructed using different conditions for the total and blank tubes, with "bound" calculated as total − blank. Linear regression in the Scatchard domain was used to estimate B_{max} and K_D values, with r^2 used to indicate the degree of fit to a linear function. The chlorpromazine-sensitive, apomorphine-insensitive [³H]fluphenazine binding is tentatively referred to as a phenothiazine (PTZ?) site. Data are mean ± SEM values of four experiments performed in triplicate.

D-1 and D-2 dopamine receptors. The resolved components resulted in linear Scatchard plots, while the aggregate component suggested some curvilinearity, although r^2 values from regression analysis were often >0.95 (Fig. 4). The curvilinearity derives from the slightly different affinities of [³H]fluphenazine for the D-1 and D-2 components. This curvilinearity also results in B_{max} values for the aggregate site that are slightly less than the sum of D-1 B_{max} and D-2 B_{max}. The line imposed by regression analysis underestimates the *x*-intercept of the curved Scatchard plot. The important feature of the saturation analyses is that using either SCH-23390 or spiperone to discriminate D-1 and D-2 components produces quantitatively similar K_D and B_{max} estimates. In addition, using a series of human caudate nucleus samples with a twofold range of D-2 B_{max} values, we obtained a high correlation between [³H]fluphenazine estimates of D-2 B_{max} and the standard [³H]spiperone estimates of D-2 B_{max} (Morgan et al., 1984*b*). The absolute B_{max} values were also similar with the two ligands. This similarity in D-2 estimates using either [³H]fluphenazine or [³H]spiperone validates the method of estimating D-2 B_{max} with [³H]fluphenazine.

We have examined the influence of age on striatal D-1 and D-2 dopamine receptors in humans. There is a gradual loss of D-2 binding sites throughout the lifespan and a concomitant increase in D-1 sites, such that the D-1 B_{max}/D-2 B_{max} ratio changes from 1 at 20 years to 3 by 80 years (Morgan et al., 1984*b*). The importance of this shift in dopamine receptor types with age in human brain is difficult to assess at present, but may predict the success of receptor-specific pharmacotherapies in age-related disorders of the basal ganglia. In any case, the selective effects of aging on the D-1 and D-2 components further support their functional distinctness.

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